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(57) Abstract

Transformed prokaryotic hosts are provided comprising two or more copies of a DNA sequence stably maintained in their chromosome, said sequence comprising a gene encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences. Methods are also provided for producing said transformed host strains. Said transformed host strains are capable of increased production of the polypeptide of interest compared to host strains which already produce said polypeptide. Preferred host strains are *Bacillus* novo species PB92 which produces a high-alkaline proteolytic enzyme and *Bacillus licheniformis* T5 which produce a thermostable alpha-amylase, and mutants and variants of said strains. Preferred polypeptide encoding genes are the protease encoding gene originating from *Bacillus* PB92 and the alpha-amylase encoding gene originating from *Bacillus licheniformis* strain T5.

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STABLE GENE AMPLIFICATION IN CHROMOSOMAL DNA OF PROKARYOTIC MICROORGANISMS

INTRODUCTION

Technical Field

The field of this invention relates to prokaryotic cells in which stable gene amplification is obtained by scattered non-tandem integration of at least two copies of a defined DNA sequence into the chromosome of said prokaryotic cell.

Background

Bacilli have been widely used for the production of industrially important enzymes such as alpha-amylase, neutral protease and alkaline or serine proteases (cf. Debabov, "The Industrial Use of Bacilli", in: The Molecular Biology of Bacilli, Acad. Press, New York, 1982). Improvement of production of Bacillus enzymes can be achieved both by classical genetic techniques, such as mutation and subsequent selection, and by modern molecular biological techniques. In the latter case, several ways of obtaining high levels of expression of homologous and heterologous genes in certain prokaryotic and eukaryotic microorganisms by genetic engineering have been well documented.

One of the approaches to achieve high level expression of a gene is to provide the gene with efficient regulatory sequences. Another approach, often used in combination with the first approach, is to increase the copy number of the gene in question. Amplification is primarily achieved by inserting the gene into a multicopy extrachromosomal DNA molecule such as a plasmid. However, a significant drawback of using plasmids as vectors for expressing and amplifying genetic information has been their instability. For large scale use, stability of the amplified gene is a prerequisite for maintaining high level production of the

expression product encoded by the amplified gene, as many cell divisions have to take place before sufficient biomass is formed for obtaining substantial product formation.

Instability is encountered in two forms: segregational instability, where loss of the plasmid occurs during cultivation; and structural instability, where a part of the plasmid is deleted. Segregational instability can occur, for example, when a host cell is harbouring a vector carrying a gene that is overexpressed. Generally there will be selective pressure towards cells that have lost the capacity to overexpress the gene, since overexpression is an unfavorable property for the transformed host cell. A large amount of metabolic energy is spent on the overexpressed gene product, which negatively influences the cells' competitiveness (growth rate) with host cells not likewise overexpressing.

A method used to counter segregational instability is to select for cells containing multicopy plasmids which carry genes which confer an advantage on the plasmid containing cell, for example, conferring resistance to an antibiotic and then to add the relevant antibiotic to the fermentation broth. However, antibiotics are generally not a useful selection means in large scale commercial production processes due to regulations concerning the approval of the fermentation process or the product itself.

Another method used to minimize plasmid loss due to segregational instability is to insert a gene which is functionally essential for the host cell into the vector molecule (Ferrari et al., Biotechnology 3 (1985) 1003-1007). However, this method does not ensure structural stability of the vector.

Techniques used to solve the problem of structural plasmid instability have included avoiding expression of the gene during the phase of exponential growth, for example, by using regulatory sequences such as temperature-sensitive regulatory sequences, and integration of the exogenous DNA into the host cell chromosome. Other methods used have included avoiding the use of autonomously replicating vector molecules and instead using techniques which favor integration

of the introduced DNA into the host cell chromosome.

Methods of achieving integration of foreign DNA into the host cell chromosome have included homologous recombination and illegitimate recombination. There are two ways of inserting DNA sequences into specific locations on a chromosome by homologous recombination: Campbell-type homologous recombination and double reciprocal recombination, which are shown in Figures 1A and 1B, respectively. A third way of introducing DNA sequences into the chromosome, this method using a two-step replacement mechanism, is shown in Figure 1C. In principle, a Campbell-type recombination is used, but the final result is a chromosomal arrangement that contains no duplicated sequences, and thus no amplifiable unit, in the recombined part of the chromosome. It therefore resembles a double reciprocal recombination.

Apart from using homologous recombination for the integration of foreign DNA into the chromosome it is also possible to integrate DNA by illegitimate recombination. Integrated vector molecules can be selected for under conditions which inhibit autonomous replication of non-integrated vector molecules. Use of illegitimate recombination for integration is depicted in Figure 1D. The absence of tandem duplications in the obtained chromosomal sequence arrangements make the pathways shown in Figures 1B, C and D preferred for stable introduction of DNA sequences into the genome. Chromosomally integrated genes have included both homologous and heterologous genes where the amplification of the chromosomally integrated DNA has been in a tandem array. These chromosomally amplified sequences have been reported to be unstable although stability has been reported in some cases. It is therefore desirable to develop methods whereby DNA integrated into the chromosome is stably maintained.

Relevant literature

Integration of exogenous DNA by homologous recombination into the chromosome of Bacillus subtilis has been described by Duncan et al., Proc. Natl. Acad. Sci. USA 75

(1978) 3664-3668 and for Anacystis nidulans by Williams and Szalay, Gene 24 (1983) 37-51 and in International Patent Application WO 84/00381. Integration by homologous recombination of a heterologous gene, which cannot be maintained stably when carried on a plasmid vector, into the chromosome of a microorganism is described in EP-A-0127328.

Amplification of chromosomally integrated genes, both homologous and heterologous has been documented. See for example: Saito et al., Proceedings of the Fourth International Symposium on Genetics of Industrial Microorganisms, Kyoto, Japan, 1982, pp. 125130; Young, J. Gen. Microbiol. 130 (1984) 1613-1621; Jannière et al., Gene 40 (1985) 47-55; Sargent and Bennett, J. Bacteriol. 161 (1985) 589-595; Gutterson and Koshland, Proc. Natl. Acad. Sci. USA 80 (1983) 4894-4898; Hashiguchi et al., Agric. Biol. Chem. 49 (1985) 545-550; Wilson and Morgan, J. Bacteriol. 163 (1985) 445-453; French Patent Application No. 84.06701; and EP-A-0134048. Spontaneous amplification in prokaryotic cells has been reported and can be selected for. See for example the review by Anderson and Roth, Ann. Rev. Microbiol. 31 (1977) 473-505.

In all cases referred to above, amplification of chromosomally integrated DNA was in a tandem array. This type of chromosomal amplification sequence has been reported to be unstable, although rather good stability was found in some cases, as discussed by Jannière et al., Gene 40 (1985) 47-55.

Stabilization of naturally occurring amplified prokaryotic genes due to the presence of other essential genes between these amplified sequences has been reported. For example, of the 9 to 10 copies of the ribosomal RNA gene sets occurring in the B. subtilis chromosome, two tandemly located sets were separated by a cluster of tRNA genes (Wawrousek and Hansen, J. Biol. Chem. 258 (1983) 291-298). In other cases, naturally occurring tandemly repeated ribosomal RNA operons were deleted, both in E. coli and in B. subtilis, with little effect on the phenotypic properties of the organism: Ellwood and Momura, J. Bacteriol. 143 (1980) 1077-1080 and Loughney et al., J. Bacteriol. 154 (1983) 529-532, respectively.

Integration of plasmids into the chromosome of B.

subtilis by illegitimate recombination using the vector pE194 has been described by Hofemeister et al., Mol. Gen. Genet. 189 (1983) 58-68 and Prorozov et al., Gene 34 (1985) 39-46.

Several genes for extracellular enzymes of bacilli have been successfully cloned, such as the alpha-amylase genes of B. amyloliquefaciens (Palva et al., Gene 15 (1981) 43-51), B. licheniformis (Ortlepp, Gene 23 (1983) 267), B. stearothermophilus (Mielenz et al., Proc. Acad. Sci. USA 80 (1983) 5975-5979; EP-A-0057976) and B. subtilis (Yang et al., Nucleic Acids Res. 11 (1983) 237); the levansucrase gene of B. subtilis (Gay et al., J. Bacteriol. 153 (1983) 1424); the neutral protease encoding g nes of B. stearothermophilus (Fuji et al., J. Bacteriol. 156 (1983) 831), B. amyloliquefaciens (Honjo et al., J. Biotech. 1 (1984) 165) and of B. subtilis (Yang et al., J. Bacteriol. 160 (1984) 115; the serine or alkaline protease encoding genes of B. subtilis (Wong et al., Proc. Natl. Acad. Sci. USA 81 (1984) 1184), B. licheniformis (Jacobs et al., Nucleic Acids Res. 13 (1985) 8913) and B. amyloliquefaciens (Wells et al., Nucleic Acids Res. 11 (1983) 7911).

Protoplast transformation for several species of gram positive microorganisms has been reported. For B. subtilis a protocol for protoplast transformation was described by Chang and Cohen (Mol. Gen. Genet. 168 (1979) 111-115), which has been widely used. Similar successful protocols have been described for the transformation of B. megaterium protoplasts (Vorobjeva et al., FEMS Microbiol. Letters 7 (1980) 261-263), B. amyloliquefaciens protoplasts (Smith et al., Appl. and Env. Microbiol. 51 (1986) 634), B. thuringiensis protoplasts (Fisher et al., Arch. Microbiol. 139 (1981) 213-217), B. sphaericus protoplasts (McDonald, J. Gen. Microbiol. 130 (1984) 203), and B. larvae protoplasts (Bakhiet et al., Appl. and Env. Microbiol. 49 (1985) 577); in the same publication unsuccessful results were reported for B. popillae. The protocol was successful for B. polymyxa, B. licheniformis, B. macerans and B. laterosporus but not for B. coagulans, B. cereus and B. pumilus, even though good protoplast formation was observed (Mann et al., Current Microbiol. 13 (1986) 131-135).

Other methods of introducing DNA into protoplasts include fusion with DNA containing liposomes (Holubova, Folia Microbiol. 30 (1985) 97), or protoplast fusion using a readily transformable organism as an intermediate host cell (EP-A-0134048).

SUMMARY OF THE INVENTION

Prokaryotic host cells, and methods for their preparation, are provided which comprise at least two copies of a DNA sequence encoding a polypeptide of interest stably integrated into the host cell chromosome. Stable maintenance of the exogenous DNA sequence is obtained by integrating two or more copies of the sequence into the host cell chromosome wherein the copies are separated by endogenous chromosomal DNA sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-D are schematic representations of four ways for integration of extrachromosomal DNA sequences into the chromosome of prokaryotic microorganisms.

T is the target sequence, i.e. DNA sequences present on chromosome and plasmid, between which homologous recombination can take place.

S stands for the DNA sequence to be integrated in the chromosome.

M stands for a marker gene sequence used for the selection of recombinant strain.

Figures 2A and 2B are schematic representations of two ways for obtaining stable gene amplification in a prokaryotic chromosome.

Figure 3 shows the results of histidine/MOPS gel electrophoresis performed on supernatant from cultures of B. subtilis DB104 containing pUB110 and pM58, respectively, compared with several subtilisins:

lane 1: Carlsberg subtilisin

lane 2: Bacillus PB92 protease

lane 3: Bacillus subtilis subtilisin
lane 4: Bacillus subtilis DB104 (pM58)
lane 5: Bacillus subtilis DB104 (pUB110)

Figure 4 shows the restriction map of plasmid pM58. Furthermore, the sequencing strategy is shown in the upper part of the figure. The arrowed solid lines represent the fragments cloned in the phage M13 vectors mp10, mp11 and mp18. The lower part of the figure shows the sequencing strategy using ten oligonucleotides located at regular distances on the protease gene.

Figure 5 shows the nucleotide sequence of the coding strand correlated with the amino acid sequence of Bacillus PB92 serine protease. Promoters (P_1, P_2), ribosome binding site (rbs) and termination regions (term) of the DNA sequence are also shown. The numbered solid lines represent the location of the ten oligonucleotides used for sequencing.

Figure 6A shows the construction of plasmid pE194-neo.

Figure 6B shows the construction of plasmid pMAX-4.

Figure 7A: Digests prepared with HindIII of chromosomal DNA of the strains PB92, PBT109 and PBT108 were subjected to electrophoresis on a 0.5% agarose gel, transferred to nitrocellulose as described by Southern and hybridized with ^{32}P labeled nick-translated pM58 DNA. The figure shows an autoradiograph.

Figures 7B and 7C illustrate the integration events occurring in case of homologous (B) recombination and illegitimate (C) recombination between pMAX-4 and the Bacillus PB92 chromosome.

Figure 8 shows the construction of integration vector pElatB.

Figure 9A illustrates the integration of plasmid pElatB into the chromosome of B. licheniformis strain T9 resulting in B. licheniformis strain TB13.

Figure 9B illustrates the chromosomal recombination of the B. licheniformis strains TB13 and T5 upon protoplast fusion of these strains, resulting in B. licheniformis strain T13F.

Figure 10 shows a chromosomal analysis of nine different colonies isolated from a fermentation of strain T13F as described in Example 11. Isolated chromosomal DNA was digested with EcoRI separated on 0.8% agarose gels and blotted onto nitrocellulose. Hybridization of the blot was performed with ^{32}P-labeled nick-translated pElatB DNA. The figure shows an autodiagram. The upper arrow indicates the position where an EcoRI DNA fragment of about 15kb migrates which contains the entire pElatB sequence that was integrated into the chromosome on a location not adjacent to the original alpha-amylase gene, as depicted for strain TB13 in Figure 9A. The lower arrow indicates the position where an EcoRI DNA fragment of about 33kb migrates which contains the entire alpha-amylase gene originally present in B. licheniformis strain T5 (see also Figure 9B). The following DNA samples were analyzed:

lane 1: Bacillus licheniformis T5 DNA
lane 2: Bacillus licheniformis TB13 DNA
lane 3: Bacillus licheniformis T390 DNA
lane 4: DNA from a neomycin-sensitive derivative of Bacillus licheniformis T390, isolated after fermentation, as described in Example 12.
lane 5: Bacillus licheniformis T13F DNA.
lanes 6-14: DNA from 9 different colonies isolated from a fermentation of strain T13F as described in Example 12.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the present invention, prokaryotic cells, and methods for their preparation, are provided in which two or more copies of a DNA sequence are stably integrated into the chromosome. A host cell comprising a DNA sequence encoding a polypeptide of interest is transformed with a DNA construct comprising said DNA sequence. Transformed cells in which the integrated DNA sequences are separated by endogenous chromosomal sequences from the gene to be amplified are then selected for. The endogenous intervening sequences

are generally vital to the host cell. Loss of amplified sequences by homologous recombination will be lethal to the host cell. Thus, there will be selection pressure for cells carrying the amplified sequences without the necessity for using antibiotics or like selection means. Integration may be achieved either by homologous recombination or by illegitimate recombination. Techniques which can be used to obtain the desired cells are as shown in Figures 2A and 2B, respectively.

When homologous recombination is used, several stretches of DNA sequences can be present in the vector molecules which are homologous to the host cell chromosome, especially when one or more copies of the gene to be amplified have already been introduced into the host cell. The vector molecule thus can include a DNA sequence of interest; a target DNA sequence; and a marker DNA sequence.

Care has to be taken that only the desired recombined chromosomal arrangements are selected for. This can be achieved by using linear DNA molecules for recombination. The circular vector molecule to be integrated is cut with a restriction enzyme in the region homologous to the target sequence. In this way recombination and integration at this specific site can occur preferentially. In addition to being present in the vector molecule, the DNA sequence of interest can also be present in the host cell chromosome. The DNA sequence may be a DNA sequence encoding any structural gene which it is desired to amplify. The DNA sequence may be endogenous to the host organism, or may have been inserted into the host chromosome in a previous transformation step.

Target sequences for non-tandem gene amplification will preferably be chosen from among non-essential genes, for example in the case of Bacilli as host organisms, the genes encoding extracellular enzymes or genes involved in sporulation can be used as target sequences. Integration of DNA sequences in these genes will generally inactivate the gene. Loss of expression of the gene can then be monitored and used for the selection of the desired recombinant strains.

When illegitimate recombination is used for chromosomal gene amplifiation as depicted in Figures 1D and

2A, conditions for integration and selection are preferred in which homologous recombination does not predominate over illegitimate recombination. A preferred means of avoiding homologous recombination is to transform first and second host cells which lack the structural gene of interest with a vector comprising a DNA sequence encoding a polypeptide of interest, and a marker gene. First and second host cells in which the DNA sequence is present at different locations can then be selected and combined under fusing conditions to yield a transformed cell with at least two copies of the DNA sequence encoding the structural gene of interest at scattered locations in the second host genome. For ease of selection the first host can be killed prior to fusion.

The gene(s) of interest may be any prokaryotic or eukaryotic gene. These genes may include bacterial genes, unicellular microorganism genes, mammalian genes, or the like. The structural genes may be prepared in a variety of ways, including synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. The various techniques of manipulation of the genes are well-known, and include restriction, digestion, resection, ligation, in vitro mutagenesis, primer repair, employing linkers and adapters, and the like. Thus, DNA sequences obtained from a host may be manipulated in a variety of ways, depending upon the requirements of the DNA construction. See Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

The structural genes may express a variety of polypeptides or proteins, such as enzymes, hormones, lymphokines, surface proteins, blood proteins, structural proteins, immunoglobulins, or the like, from mammals, unicellular microorganisms, e.g., bacteria, fungi, such as yeast, or filamentous fungi, algae, protozoa, etc., plants or other DNA source. Of particular interest are enzymes, more particularly proteases and amylases. Illustrative of such enzymes are serine and non-serine proteases, including high alkaline serine proteases, alpha- and bêta-amylase, and the like. A preferred source for a serine protease is Bacillus novo species PB92, and for an

alpha-amylase is B. licheniformis strain T5, as well as mutants and variants of these strains.

The gene that forms part of the suitable vector can be obtained by methods generally known in the art. Generally, the method comprises preparing a genomic library from the organism expressing a high alkaline protease. The genomic library is conveniently prepared for example by ligating DNA fragments of the donor strain into a suitable vector.

By the term "suitable vector" is meant a DNA construct comprising a structural gene encoding a protein of polypetide of interest. The structural gene is joined in proper orientation to control regions such as a promoter sequence, a sequence forming the ribosome binding site and sequences controlling termination of transcription and translation of the structural gene, which control regions are functional in the host cell. Where the host cell has transformation and integration frequencies which are too low to permit direct selection for integration without intermediate isolation of plasmid containing cells, such as industrial Bacillus strains, the vector can additionally comprise an origin of replication that is capable of replicating autonomously in the host cell.

Where the gene is obtained from a donor cell which has transcriptional and translational initiation and termination regulatory signals which are recognized by the host prokaryotic cell strain, it will usually be convenient to maintain the original regulatory sequences of the structural gene. In addition, the transcriptional initiation region may provide for constitutive or inducible expression, so that in appropriate situations, the host may be grown to high density before high levels of expression of the structural genes of interest are obtained.

Where the structural gene is from a source whose regulatory signals are not recognized by the host cell, it will be necessary to obtain regulatory regions recognized by the host cell and to insert the structural gene between the initiation and termination regulatory signals. In some instances the exogenous structural gene with its own stop

codon(s) may be inserted in reading frame behind the N-terminus codons of an endogenous structural gene which retains its natural regulatory signals.

It is desirable that the expression product be secreted. Where the expression product is naturally secreted and the leader signals and processing signal(s) are recognized by the host cell, this will entail no difficulty. However, where the product is not secreted because the host cell does not recognize the secretory leader signals and/or processing signal(s), or the signals are not functional to a satisfactory degree in the host cell, then it may be necessary to isolate or synthesize DNA sequences coding for the secretory leader signals and processing signal(s) of a host cell polypeptide and join them in proper reading frame to the 5'-end of the structural gene.

The vector may additionally include a marker gene conferring resistance to an antibiotic to which the host strain is sensitive. The marker gene, when used in chromosomal integration of the vector, has to fulfill the demand that survival selection is possible even if only one or a few copies of the marker gene are present in the host strain. By marker is intended a structural gene capable of expression in a host, which provides for survival selection. By "survivial selection" is intended imparting prototrophy to an auxotrophic host, biocide or viral resistance. For prototrophy, various genes may be employed, such as leu, his, trp, or the like. For biocide resistance this may include resistance to antibiotics, e.g., neo, cam, tet, tun, kan, or the like. Other markers include resistance to heavy metals, immunity, and the like. The various DNA sequences may be derived from diverse sources and joined together to provide for a vector which includes one or more convenient, preferably unique, restriction sites to allow for insertion or substitution of the structural genes at such sites or in place of lost fragments to provide the plasmid construct.

Selection for chromosomal integration may be aided by using a plasmid with an origin replication having a mutation which makes its functioning temperature-sensitive in

the host cell. See, for example, Ehrlich, Proc. Natl. Acad. Sci. USA 75 (1978) 1433.

Once the plasmid construct has been prepared, it may now be cloned in an appropriate cloning host. Any host may be used which is convenient, is readily transformable, and allows for replication of the plasmid construct and transfer to the host cell. A large number of strains are available which have a high efficiency of transformation and are usually auxotrophic and/or antibiotic sensitive. Where the host cell is an industrial Bacillus strain, the use of the same organism as the host cell for cloning of the plasmid construct has many advantages in that it permits the use of a single replication system as well as the same marker for survival selection in both the cloning host and the host strain. See, for example, European application EP-A-134048, which disclosure is incorporated herein by reference.

The plasmid construct may be introduced into the cloning host in accordance with conventional techniques, such as transformation, employing calcium precipitated DNA, conjugation, or other convenient technique. The cloning host may then be grown in an appropriate nutrient medium, under selective conditions to select for a host containing the plasmid construct. For auxotrophic hosts, the nutrient medium is deficient in the required nutrient, while for biocide resistance, e.g., antibiotic resistance, a cytotoxic amount of the biocide(s) is employed in the nutrient medium.

Various host cells may be employed. These include E. coli, Bacillus strains, expecially Bacillus subtilis, Pseudomonas, and Streptomyces. In choosing a host cell, various factors are taken into account, including factors which can affect expression of the gene to be amplified and production of the desired product. Thus it is desirable to use a host cell in which there is recognition of regulatory signals; ease of secretion; reduced degradation of the desired product, etc. A preferred host cell already produces the polypeptide of interest, and may be either a wild type organism or a mutant organism. The host cell can also be a mutant of an organism which produces the polypeptide of

interest which itself, however, is non-producer. Where the polypeptide of interest is a protease or an amylase, preferred strains include Bacillus novo species PB92 and Bacillus licheniformis strain T5, respectively, as well as mutants and variants of these strains.

In addition, industrial strains may be employed which have the desired traits of an industrial strain. Examples of strains which may be employed include strains used for the industrial production of enzymes such as: B. licheniformis, B. amyloliquefaciens and alkalophilic Bacilli. The industrial strains are chosen from organisms which may be isolated in the soil or available from depositories or other sources or obtained by modification of such strains. The industrial strains are highly robust and stable. Furthermore, said strains are resistant to phage infection and to genetic exchange, that is introduction of DNA by conventional transformation procedures. The conventional industrial strains are also prototrophic, in order to avoid adding expensive amino acids to the nutrient medium. Other characteristics of industrial strains are their high productivity until the end of the fermentation, which can be as long as a week, stable cell concentration upon exhaustion of the broth, and high productivity, usually at least 5 g/l (0.5% w/v) of a specific secreted protein.

Transformants can be obtained having genes either in tandem arrangement or scattered in the chromosome. In general, it is possible to select transformants containing scattered genes from mixtures of the two types of transformants mentioned, by isolating chromosomal DNA of each individual transformant, subsequently analyzing said DNA with respect to the relative locations of said genes by, for example, the method of Southern, J. Mol. Biol. 98 (1975) 503-517 or other means known to those skilled in the art, thereby identifying scattered integration of said genes.

Means for obtaining scattered gene transformants avoiding tandem integration include, using double reciprocal recombination as illustrated in the Figure 1B and 2B, the use of linearized DNA constructs comprising the DNA sequence to be

amplified, a marker gene and target sequences for recombination.

Furthermore, specific means for obtaining scattered gene transformants include the use of illegitimate recombination as illustrated in Figure 2A, in which isolation of tandem transformants can be avoided by selection, using differential expression of a marker gene, for example a gene encoding antibiotic resistance, where sensitivity to the antibiotic is different in strains with tandem integration of the gene as opposed to non-tandem integration. Generally, the length of the intervening endogenous DNA sequences will be less than 10 kbp.

Additionally, means for obtaining scattered gene transformants avoiding tandem duplication include the use of killed protoplasts of a homologous donor strain carrying a DNA construct comprising the structural gene and a marker gene, the structural gene being integrated in the chromosomal at a different location with respect to the acceptor strain.

Transformation of the host cells preferably involves the use of protoplasts prepared from the host strain. Protoplasts generally are prepared from the cells in accordance with conventional ways, e.g. lysozyme or zymolyase treatment, and the protoplasts carefully suspended in an appropriate medium having proper osmolalities for maintaining the integrity of the protoplast. For industrial Bacillus strains, methods for preparing protoplasts are described in EP-A-0134048, which disclosure is incorporated herein by reference. Where the host strain is an alkalophilic Bacillus strain, protoplasts may conveniently be prepared at alkaline pH, preferably about pH 8.0. This procedure is disclosed in European Application No. EP-A-87200358.7, which disclosure is incorporated herein by reference.

The host cell can be transformed by combining the plasmid construct or a cloning host protoplast with the host cell protoplast in the presence of an appropriate fusogen. Any fusogen may be employed which provides the desired degree of efficiency, for the most part polyethylene glycol is found to provide high efficiency of fusion with great convenience.

After a short time, the fusogen mixture is replaced with an appropriate nutrient medium and cells regenerated in a selective medium, conveniently by plating on an agar plate.

Transformants obtained by combining a host cell with a suitable DNA construct can contain said DNA construct or part thereof either directly as an integral part of their chromosome or as free vector molecules when the DNA constructs contain an origin of replication functional in said host cell.

A means of selecting for transformants wherein the DNA construct is integrated into the chromosome is to use a plasmid containing a temperature-sensitive origin of replication. Transformants are grown in a selective medium at the permissive temperature, then shifted to a non-permissive temperature. Colonies expressing the marker gene at the non-permissive temperature are then isolated and cultured in selective medium at the permissive temperature. Plasmid absence can be verified, for example, by isolating total DNA from the colonies and electrophoresising on an agarose gel or by demonstrating lack of ability of the transformants to transform competent cells. Determination of the way in which integration into the chromosome has taken place can be analysis of the chromosomal DNA by, for example, the method of Southern, J. Mol. Biol. 98 (1975) 503-517 or other means known to those skilled in the art.

When there is a differential sensitivity to the selective agent between transformants containing additional copies of the marker gene in a tandem array as compared to those in which the marker gene is incorporated at scattered locations in the host genome, transformants can conveniently be grown in medium containing the appropriate concentration of selective agent to select for transformants with non-tandem integration.

Another means of obtaining transf rmants with scattered integration of copies of the DNA sequence of interest is to use a protoplast prepared from a homologous donor cell, containing at least one copy of the DNA sequence of interest at a location on its chromosome different from that of the recipient host cell. The homologous donor cell can

be prepared, for example, by transforming a cell which does not contain the structural gene of interest with a vector comprising the structural gene. Integration of the DNA sequence into the donor cell chromosome can be facilitated by using a plasmid containing a temperature-sensitive origin of replication and growing transformants under selective conditions first at the permissive temperature and then at the non-permissive temperature as described above, then isolating colonies expressing the marker gene.

Following verification of the absence of plasmid DNA, the chromosomal DNA can be isolated and analyzed according to the method of Southern, supra, by hybridizing with a probe labeled with, for example, ^{32}P or biotinylated nucleotides. The probe may be cDNA encoding the polypeptide of interest or fragments thereof as well as DNA constructs or fragments thereof comprising the DNA sequence of interest, for example a vector. Transformants containing the gene of interest at an alternate location as compared to that of the gene donor strain can then be used as an homologous donor cell. The recipient strain host is preferably the same as the strain used as the source of the DNA sequence of interest, or a strain in which the DNA sequence of interest is located at a different region of the chromosome than in the transformed donor cell.

To aid in selection, the donor cell preferably is killed with a cytotixic agent prior to or during protoplast formation. Various agent may be employed to kill the donor cell, including antibiotics, but iodoacetamide is found to be convenient, efficient, and does not interfere with the subsequent fusion. When dead cloning host protoplasts are used, the ratio of dead protoplast to the acceptor strain host will be preferably at least about 1:1 and an excess of the dead protoplast may be employed.

Following fusion of the dead donor cell protoplast and the recipient host cell protoplast, transformants can be selected by means of the marker gene. DNA can then be isolated and analyzed as described above to identify transformants in which more than one copy of the gene of interest has been

incorporated into the genome and are separated by endogenous chromosomal sequences.

Scattered two-gene transformants are then screened in appropriate ways for detection of increased expression of the polypeptide of interest. Various techniques may be employed, particularly where enzymes are involved which have well established methods of detection. Alternatively, where enzymes are not involved or there is no available detection system, bioassays, antibodies, or DNA or RNA hybridization can be employed for screening the clones to determine the presence of the plasmid construct and expression of the structural gene of interest.

The host cell containing the chromosomally integrated plasmid constructs or fragments thereof is then grown in a nutrient medium under conventional fermenting conditions. The fermenting may be continued until the broth is exhausted. Where the product has been secreted, the product may be isolated from the broth by conventional techniques, e.g., extraction, chromatography, electrophoresis, or the like. Where the product is retained in the cytoplasm, the cells may be harvested by centrifugation, filtration, etc., lysed by mechanical shearing detergent, lysozome, or other techniques and the product isolated as described previously. By employing the subject method, stable integration of at least two copies of a DNA sequence can be achieved as a means of gene amplification.

The following examples are offered by way of illustration and nor by way of limitation.

EXPERIMENTAL

EXAMPLE 1

Preparation of a Genomic DNA Library from Alkalophilic Bacillus novo sp. PB92 and Isolation of the Serine Protease Gene

Chromosomal DNA isolated from Bacillus novo sp. PB92 (deposited under No. OR-60 with Laboratorium voor Microbiologie, Technical University of Delft, the Netherlands, see U.S. Patent No. Re. 30,602) according to the procedure described by Saito-Miuva, Biochim. Biophys. Acta 72 (1963) 619-632, was partially digested with the restriction enzyme Sau3A and ligated into the BamHI site of plasmid pUB110 (Gryczan et al., J. Bacteriol. 134 (1978) 318-329). pUB110 plasmid DNA was prepared as described by Birnboim and Doly (Nucl. Acids Res. 7 (1979) 1513-1523).

The ligation mixture was transformed into B. subtilis 1A40 (Bacillus Genetic Stock Centre) according to the method of Spizizen et al., J. Bacteriol. 81 (1961) 741-746, using 0.6-1 µg DNA per ml of competent cells. Cells from the transformation mixture were plated on minimal plates containing: 2.8% K_2HPO_4, 1.2% KH_2PO_4, 0.4% $(NH_4)_2SO_4$, 0.2% tri-Na-citrate.$2H_2O$, 0.04% $MgSO_4.7H_2O$, 0.00005% $MnSO_4.4H_2O$, 0.4% L-glutamic acid, 0.5% glucose, 0.02% casamino acids, 50 µg/ml trypthophan, 20 µg/ml methionine, 20 µg/ml lysine, 20 µg/ml neomycin, 0.4% casein and 1.5% agar. After overnight incubation of the plates at 37°C one out of 50,000 neomycin resistant colonies showed increased protease production, as determined by increased precipitation of a halo of casein cleavage products around the colony in the agar plate. Plasmid DNA was isolated from this colony according to the method described by Birnboim and Doly, Nucleic Acids Res. 7 (1979) 1513-1523, and named pM58.

EXAMPLE 2

Expression of the PB92 Serine Protease Gene

Bacillus subtilis 1A40 containing pM58 was grown in minimal medium (Spizizen et al., Proc. Natl. Acad. Sci. USA 44 (1958) 1072-1078) to which had been added 0.02% casamino acids 50 µg/ml trypthophan, 20 µg/ml methionine, 20 µg/ml lysine and 20 µg/ml neomycin. After 24 hours, the culture was centrifuged and the supernatant assayed for protease activity using dimethyl casein as substrate (Lin et al., J. Biol. Chem. 244 (1969) 789-793. A culture of B. subtilis 1A40 containing the plasmid pUB110 used as a control showed less than 1/60 of the protease activity shown by the pM58 transformed culture. Protease activity was completely inhibited by treatment with 1 mM phenylsulfonyl fluoride (PMSF), but not by treatment with 20 mM EDTA.

Aliquots of the above described supernatants were analyzed on protein gel according to the method of Laemmli, Nature 227 (1970) 680. Samples for analysis on these gels were prepared by treatment of the supernatants with 5% trichloroacetic acid (TCA). Following centrifugation of the sample the pellet of precipitated protein was washed twice with acetone then dissolved in 40 µl sample buffer (0.5 M Tris/HCl pH 7.5, 10% v/v 2-mercaptoethanol, 50% v/v glycerol and 0.05% Bromophenol Blue) by boiling for 10 minutes. After electrophoresis, the gels were stained using Coomassie Brilliant Blue. Culture supernatant samples were then analyzed by electrophoresis. Three different B. subtilis 1A40 strains were used: a strain containing pUB110; or pM58; or no plasmid; and Bacillus PB92 protease as a control. After electrophoresis, the gels were stained using Coomassie Brilliant Blue and destained. The sample from B. subtilis strain 1A40 containing pM58 contained a 31 kD protein, which comigrates with Bacillus PB92 protease. This protein was not detected on the control lane of strain B. subtilis 1A40 containing pUB110.

All serine proteases have similar molecular weights. The cloned serine protease of Bacillus PB92 therefore

was differentiated from known serine proteases (B. subtilis subtilisin, Carlsberg subtilisin), by transformation of pM58 and pUB110 to the protease negative B. subtilis strain DB104 (R. Doi, J. Bacteriol. 160 (1984) 442-444) and analysis of the extracellular proteases produced. The obtained transformants were grown in minimal medium (Spizizen et al., supra) containing 0.02% casamino acids, 50 µg/ml histidine and 20 µg/ml neomycin. After 24 hours, samples were taken, centrifuged and without pretreatment analysed on histidine/MOPS gels containing 75 mM KOH, 40 mM histidine, 100 mM MOPS (3-(N-morpholino)-propanesulfonic acid), pH 7.5 and 5% polyacrylamide. Electrophoresis buffer contained 40mM histidine, 100 mM MOPS, pH 6.6. Samples were run in the direction of the cathode. Protease bands were detected with Agfa Pan 100 Professional films (Zuidweg et al., Biotechnol. and Bioengin. 14 (1972) 685-714). These results are shown in Figure 3. As shown in Figure 4, pM58 harbours the gene encoding Bacillus PB92 protease.

EXAMPLE 3

Sequencing of the Bacillus PB92 Serine Protease Gene

The entire sequence of a BalI-HpaI fragment of pM58 was determined by the method of Sanger, Proc. Natl. Acad. Sci. USA 74 (1977) 6463. Restriction fragments of pM58 (see Figure 4) were cloned in phage M13 vectors mp10, mp11 and mp18 (Messing et al., Nucleic Acids Res. 9 (1981) 309-321. Insertions of pM58 fragments were screened by plaque hybridization. After sequencing, ten oligonucleotides located at regular distances on the gene were made and sequencing was repeated, confirming the sequence shown in Figure 5.

EXAMPLE 4

Construction of Serine Protease Containing Plasmid pMAX-4

To construct plasmid pUCN710 (Figure 6A) pUB110 was digested with TaqI and PvuII. The fragment containing the gene conferring neomycin resistance was purified on low melting agarose and made blunt with Klenow polymerase and NTP's (Maniatis, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor 1982). Plasmid pUC7 (Vieira et al., Gene 19 (1982) 259-268) was linearized with SalI and made blunt as described above. Both fragments were ligated with T4 ligase (Maniatis) and transformed to E. coli JM103. Selection took place on 2xTY plates (1.6% w/v Bacto-trypton, 1% w/v yeast extract, 0.5% NaCl) containing 50 µg/ml ampicillin and 10 µg/ml neomycin. The resulting plasmid, named pUCN710, was digested with BamHI. The plasmid pE194 (Jordanescu, Plasmid 1 (1978) 468-479) was digested with BclI. The fragments from both digestions were ligated with T4 ligase and transformed to B. subtilis 1A40. Selection took place on minimal plates containing 20 ug/ml neomycin (see Example 1). The plasmid obtained, pE194-neo (Figure 6A) contains the neomycin gene and a temperature sensitive origin of replication.

Subcloning of the protease gene in integration vector pE194-neo was performed as follows: pM58 (see Example 1) was digested with HpaI and BalI and BglII. Plasmid pE194-neo was digested with HpaI. These fragments were ligated with T4 ligase and transformed to B. subtilis 1A40. Transformants were selected based upon neomycin resistance and an increase in protease production, as judged by casein cleavage products precipitation (halo formation, see Example 1). Plasmid pMAX-4 was obtained, the structure of which was confirmed by restriction enzyme analysis (see Figure 6B).

EXAMPLE 5

Protoplast Transformation of
Bacillus Strain PB92 by pMAX-4

Bacillus strain PB92 was grown overnight in 100 ml NBSG-X medium (Thorne et al., J. Bacteriol. 91 (1966) 1012-1020). The culture was centrifuged for 10 minutes at 4,500 rpm in a Sorvall model GSA rotor. Protoplasts were prepared by incubating the bacilli for one hour at 37°C in 10 ml Alkalic Holding Medium (AHM) containing 0.5 M sucrose, 0.02 M $MgCl_2$ and 0.02 M Tris/maleate, pH 8.0, in sterile water to which 0.4 mg/ml lysozyme was added. The protoplasts were pelleted (10 minutes at 4,500 rpm), resuspended in 5 ml AHM^+ pH 8.0 buffer (AHM buffer to which 3.5% w/v Bacto Penassay Broth and 0.04% w/v Albumine Merieux had been added) mixed, then repelleted as above. After being resuspended in 5.0 ml of alkaline holding medium, 0.5 ml of this suspension of protoplasts were mixed with 5 µg of demineralized water containing 1 µg of plasmid DNA and incubated for 2 minutes in the presence of 30% w/v polyethylene glycol 8,000, pH 8.0. After 1:3 dilution with AHM^+ pH 8.0 medium and centrifugation, the pellet was re-suspended in a small volume (1 ml) of AHM^+ and incubated for 2-3 hours. One hundred microliter aliquots were plated on freshly prepared regeneration plates containing 0.5 M Na succinate/HCl pH 8.0, 1.5% w/v agar, 0.5% w/v casamino acids, 0.5% w/v yeast extract, 0.031 M phosphate buffer pH 8.0, 0.5% w/v glucose, 0.02 M $MgCl_2$ and 0.02% w/v Albumine Merieux. These plates also contained 1000 µg/ml neomycin for selection. After incubation at 37°C for at least 72 hrs, the colonies were replica-plated onto heart infusion agar plates containing 20 µg/ml neomycin.

EXAMPLE 6

Integration of pMAX-4 in the
Bacillus Strain PB92 Chromosome

A transformant of Bacillus PB92, containing plasmid pMAX-4, was incubated in Tryptone Soya Broth (TSB) containing either 1 µg/ml or 20 µg/ml neomycin for 24 hrs at 37°C. Two ml portions of the cell suspensions were then diluted in 100 ml of TSB containing 1 µg/ml or 20 µg/ml neomycin, respectively, and incubated for 24 hrs at 50°C. After 24 hrs 5 ml samples of both cultures were diluted again, as described above, and incubated for 24 hrs at 50°C, again in the presence of 1 µg/ml or 20 µg/ml neomycin, respectively. The last procedure was repeated once more. The cell suspensions were then diluted 100-fold and plated on Heart Infusion (HI) agar plates containing 1 µg/ml neomycin for the samples from the flasks containing 1 µg/ml neomycin, and 20 µg/ml neomycin for the samples from the flasks containing 20 µg/ml neomycin. The plates were incubated for 16 hrs at 50°C. Neomycin-resistant colonies were isolated and cultured in 10 ml TSB medium containing 1 µg/ml neomycin for 16 h at 37°C. From these cultures total DNA was isolated (Holmes et al., Anal. Biochem. 114 (1981) 193-197. Plasmid absence was verified by DNA electrophoresis on agarose gel. Absence of plasmid DNA from samples in which plasmid DNA was not detectable was confirmed by transformation of total DNA to B. subtilis 1A40. Samples lacking the ability to transform B. subtilis 1A40 were considered plasmid-free.

To check whether and in what way integration of pMAX-4 in the chromosome took place, chromosomal DNA was isolated, digested with HindIII, run on 0.5% DNA agarose gels and blotted to nitrocellulose (Southern, J. Mol. Biol. 98 (1975) 503-517), and hybridized with ^{32}P labeled nick-translated pM58 (Maniatis, 1982). The result of this analysis is shown in Figure 7A.

Selection at 1 µg/ml neomycin resulted in protease genes tandemly located in the chromosome and separated by plasmid sequences (strain PBT109) as a result of homologous recombination (Campbell-type mechanism). In an accumulation of 30 independently isolated integrants, selection was performed at 1 µg/ml neomycin. One integrant was isolated which contained the plasmid pMAX-4 on a random location in the

chromosome as a result of an illegitimate recombination (strain PBT122). Selection at 20 µg/ml neomycin resulted in a copy of plasmid pMAX-4 on a random location in the chromosome as a result of an illegitimate type of recombination. The latter strain was named PBT108. The genetic organization of the strains PBT109 and 108 are depicted in Figures 7B and 7C, respectively. Chromosomal analysis showed that integration in PBT122 and PBT108 occurred on different locations in the chromosome.

EXAMPLE 7

Stability of the Duplicated Protease Genes in Strains PBT108 and PBT 109

One hundred ml of production medium (containing: 1% starch, 4% lactose, 0.87% K_2HPO_4, 0.5% yeast extract, 0.5% $(NH_4)_2HPO_4$, 0.2% Tri Na citrate.$2H_2O$, 0.05% $MgSO_4.7H_2O$, 0.07% $CaCl_2$, 0.068% $FeSO_4.7H_2O$ and antifoam 1 ml/l) without neomycin was inoculated with 0.2 ml of an overnight TSB culture (37°C) of strain PBT108 or PBT109 in 500ml shake flasks. After incubation for 44 hrs at 37°C under constant aeration the culture was tested for neomycin-resistant colonies and for protease activity.

Both strains PBT108 and PBT109 were also tested in Eschweiler fermenters containing the same production medium to check the effect of upscaling to 10 l. The results of the fermentation experiments are summarized in the following Table 1.

Table 1

Strain	Relative Production of Protease*	Percent of Neomycin-Resistant Cells After Fermentation
Control (PB92)	100%	-
PBT108	120%	100%
PBT109	115-120%	75-97%

* Protease activity was assayed using dimethylcasein as substrate as described by Lin et al., J. Biol. Chem. 244 (1969) 789-793.

Analysis of colonies derived from the Eschweiler fermentation of PBT109 after 2 days of culturing, showed that 3-25% of these colonies produced at the level of a strain containing only a single protease gene. These same colonies were found neomycin-sensitive due to excission of the pMAX-4 sequence by homologous recombination. However, analysis of the colonies derived from the strain PBT108 fermentation experiment showed that these cells were all neomycin-resistant. One hundred of these neomycin-resistant colonies were taken at random and individually tested for protease production potential, to determine whether they contained one or two productive protease genes. All 100 individually tested colonies produced at the level of a strain containing two genes, showing that the two randomly integrated protease genes in PBT108 are stably maintained under the fermentation conditions used.

EXAMPLE 8

Construction of Integration Vector pElatB

Plasmid pGB34, described in EPA 0134048, was digested with the restriction enzymes BclI, BglI and BglII. The restriction fragments obtained were blunt-ended with Klenow polymerase, then were ligated into the HpaI site of pE194-neo (see Example 6). Plasmid pE194-neo DNA was isolated as described by Birnboim and Doly (Nucl. Acids. Res. 7 (1979) 1513-1523).

The ligation mixture was transformed into B. subtilis 1-A40, according to the method of Spizizen et al (J. Bacteriol. 81 (1961) 741-746) using 0.5-1 µg DNA per ml of competent cells. Cells from the transformation mixture were plated on minimal plates containing 2.8% K_2HPO_4, 1.2% KH_2PO_4, 0.4% $(NH_4)_2SO_4$, 0.2% Tri Na citrate.$2H_2O$, 0.04% $MgSO_4.7H_2O$,

0.00005% $MnSO_4.4H_2O$, 0.4% glutamic acid, 0.5% glucose, 0.02% casamino acids, 50 µg/ml tryptophan, 20 µg/ml methionine, 20 µg/ml lysine, 20 µg/ml neomycin, 0.4% casein, 0.5% starch and 1.5% agar.

DNA of alpha-amylase producing colonies was isolated as described by Birnboim and Doly and checked with restriction enzymes. From one of these transformants plasmid pElatB, see Figure 8, was isolated.

EXAMPLE 9

Transformation of the Alpha-Amylase Negative Strain Bacillus licheniformis T9 with pElatB

Transformation of Bacillus licheniformis strain T9 was carried out as described in EP-A-0253455 with the exception that the entire procedure was performed at 30°C instead of 37°C. Selection for transformants was carried out on minimal plates containing 20 µg/ml neomycin. All transformants produced amylase. Restriction enzyme analysis performed on DNA prepared as described by Birnboim and Doly showed that the transformants all contained pElatB.

EXAMPLE 10

Integration of pElatB into the B. licheniformis T9 chromosome

Bacillus licheniformis strain T9 containing plasmid pElatB, was inoculated in Tryptone Soya Broth (TSB) containing 20 µg/ml neomycin and incubated for 16 hours at 30°C. A 5 ml portion of the cell suspension was diluted in 100 ml of the same medium and incubated at 50°C for 24 hours.

This procedure was repeated once. The cell suspension was then diluted 100-fold and plated on Heart Infusion Agar plates containing 10 µg/ml neomycin. After 40 hours of incubation at 50°C, neomycin-resistant colonies were isolated and cultured in 10 ml TSB medium, containing 10 µg/ml

neomycin, for 16 hours at 30°C. Total DNA from these cultures was isolated (Holmes et al., Anal. Biochem. 114 (1981) 193-197). The absence of plasmids in these cells was verified by DNA electrophoresis on agarose gels. Samples in which low molecular weight DNA was virtually absent, were rechecked on the presence of plasmid DNA by DNA transformation to B. subtilis 1-A40 (Spizizen et al., 1961). Samples lacking the ability to transform B. subtilis 1-A40 to neomycin resistance were considered plasmid minus.

To check whether integration of pElatB took place and how it took place, chromosomal DNA was isolated from the transformants (Saito-Minwa, Biochem. Biophys. Acta 72 (1963) 619-632), digested with EcoRI fractionated on 0.5% agarose gels, blotted onto nitro-cellulose (Southern, J. Mol. Biol. 98 (1975) 503-517) and hybridized with ^{32}P labeled nick-translated pGB33 (see EP-A-0134048). The results from this analysis are shown in Figure 9A. The data show that illegitimate recombination of pElatB took place resulting in a strain containing a single amylase gene on a different locus of the genome as compared with the original Bacillus licheniformis T5 amylase strain. The strain obtained containing pElatB was named TB13.

EXAMPLE 11

Construction of Strain T13F Containing Two Amylase Genes Separated by Endogenous Chromosomal Sequences

In order to develop a strain containing two amylase genes separated by endogenous chromosomal DNA sequences, a fusion experiment was performed between Bacillus licheniformis strain T5 (the original amylase gene containing amylase strain, see EP-A-0134048) and strain TB13 (the randomly integrated, amylase gene containing strain). Protoplast fusion was performed as described in EP-A-0134048, the disclosure of which is hereby incorporated by reference. Strain TB13 was killed with iodoacetamide prior to protoplast formation. Strain T5 (neomycin sensitive) was not killed. Selection for

fusants took place on the regeneration plates containing 10 ug/ml neomycin.

To check and identify potential fusants, chromosomal DNA was isolated, digested with EcoRI, fractionated on 0.5% agarose gels, blotted to nitrocellulose filters (Southern, J. Mol. Biol. 98 (1975) 503-517) and hybridized with ^{32}P labeled nick-translated pGB33 (see EP-A-0134048). The result of this analysis is shown in Figure 9B. One of the obtained fusants, T13F, contained two amylase genes separated by endogenous chromosomal sequences.

EXAMPLE 12

Stability of the duplicated amylase genes in strains T390 and T13F

The stability of strain T13F, a strain containing two chromosomal amylase genes separated by essential chromosomal sequences, was compared with that of strain T390, a strain with two chromosomal amylase genes located in a tandem array. Preparation of strain T390 is disclosed in EP-A-134048 (page 17, Table I), where it was referred to as B. licheniformis T5 (pGB33). Strains T13F and T390 were tested under fermentation conditions, nameley 0.2 ml of an overnight TSB culture (37°C) was inoculated in 500 ml shake flasks containing 100 ml production medium (see Example 7; after sterilization the pH was adjusted to 6.9 with NaOH) without neomycin. After incubation for 6 days at 40°C under constant aeration the culture was tested for neomycin-resistant colonies and amylase activity. The results of the fermentation experiments are summarized in the following Table 2.

Table 2

Strain	Relative Amylase Activity	Percent of Neomycin-Resistant Cells After Fermentation*
T5	100%	-
TB13	20%	100%
T13F	120%	100%
T390	200%	88%

* More than thousand colonies were tested per strain.

To exclude the possibility of excission of one amylase gene without concomitant loss of the neomycin gene in strain T13F, 20 colonies derived from T13F fermentation were analyzed. Chromosomal DNA from 20 randomly chosen colonies was isolated and characterized by hybridization experiments as described above. The results of 9 of these analyses are shown in Figure 10. All strains tested contained two amylase genes, as demonstrated by the presence of two alpha-amylase genes containing EcoRI fragments in their chromosomal DNA.

In contrast to the genetic stability of strain T13F, strain T390 was found to be unstable upon fermentation resulting in 12% neomycin-sensitive colonies. One of these colonies was analyzed and found to contain only one alpha-amylase gene (Figure 10, lane 4). This shows that randomly integrated amylase genes are more stable than tandemly integrated genes, under fermentation conditions.

It is evident from the above results that a prokaryotic cell may be obtained in which stable gene amplification is achieved by selecting for transformed cells in which non-tandem integration of at least two copies of the structural gene to be amplified has occurred. Integration may occur by homologous recombination or illegitimate recombination.

All publications and patent applications mentioned in this specification are indicative of the level of skill of

those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A transformed prokaryotic host cell comprising at least two copies of a DNA sequence in its chromosome, said DNA sequence encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences.

2. A transformed prokaryotic host cell comprising at least two copies of a DNA sequence encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences in the genome of said host cell, said cell being produced by the method comprising:

combining a recipient host cell comprising at least one copy of said DNA sequence integrated into its chromosome with (a) a DNA construct, comprising at least one copy of said DNA sequence and at least one of a marker gene and a temperature-sensitive origin of replication or (b) a donor host cell comprising said DNA construct, under transforming conditions;

selecting for a transformant wherein said DNA construct is integrated into the chromosome of said transformant; and

isolating from among said transformants, transformed prokaryotic host cells comprising at least two copies of said DNA sequence separated by endogenous DNA sequences.

3. A transformed prokaryotic host cell according to Claim 1 or 2, wherein said prokaryotic cell is a Bacillus strain.

4. A transformed prokaryotic host cell according to Claim 3, wherein said Bacillus strain is an alkalophilic Bacillus strain or a Bacillus licheniformis host strain.

5. A transformed prokaryotic host cell according to Claim 4, wherein said alkalophilic Bacillus strain is Bacillus novo species PB92 or a mutant or variant thereof.

6. A transformed prokaryotic host cell according to Claim 4, wherein said Bacillus licheniformis host strain is Bacillus licheniformis strain T5 or a mutant or a variant thereof.

7. A transformed prokaryotic host cell according to Claim 1 or 2, wherein said polypeptide of interest is an enzyme.

8. A transformed prokaryotic host cell according to Claim 7, wherein said enzyme is a proteolytic enzyme or an amylolytic enzyme.

9. A transformed prokaryotic host cell according to Claim 8, wherein said proteolytic enzyme is a serine protease.

10. A transformed prokaryotic host cell according to Claim 9, wherein said serine protease comprises substantially the following amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH.

11. A transformed prokaryotic host cell according to Claim 8, wherein said amylolytic enzyme is alpha-amylase.

12. A transformed prokaryotic host cell according to Claim 1 or 2, wherein the genome of Bacillus novo species PB92 or a mutant or variant thereof comprises said DNA sequence.

13. A transformed prokaryotic host cell according to

Claim 1 or 2, wherein the genome of Bacillus licheniformis strain T5 or a mutant or a variant thereof comprises said DNA sequence.

14. A method for preparing a transformed prokaryotic host cell comprising at least two copies of a DNA sequence encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal sequences in the genome of said host cell, said method comprising:

combining a recipient host cell comprising at least one copy of said DNA sequence integrated into its chromosome with (a) a DNA construct, comprising at least one copy of said DNA sequence and at least one of a marker gene and a temperature-sensitive origin of replication or (b) a donor host cell comprising said DNA construct, under transforming conditions;

selecting for a transformant wherein said DNA construct is integrated into the chromosome of said transformant; and

isolating from among said transformants, transformed prokaryotic host cells comprising at least two copies of said DNA sequence separated by endogenous DNA sequences.

15. A method according to Claim 14, wherein said selecting comprises:

growing said transformant comprising a DNA construct comprising a marker gene in the presence of a biocide to which said marker gene provides resistance; and

identifying and isolating from said transformants, plasmid free transformants.

16. A method according to Claim 14, wherein said selecting comprises:

growing said transformant comprising a DNA construct comprising a marker gene and a temperature-sensitive origin of replication in the presence of a biocide at a non-

permissive temperature; and

selecting from said transformants, plasmid free transformants.

17. A method according to Claim 14, wherein said isolating comprises:

isolating chromosomal DNA from said transformants; and

hybridizing said chromosomal DNA with a labeled probe comprising said DNA construct or a fragment thereof whereby said transformed prokaryotic host cells are selected by detecting said label.

18. A method according to Claim 14, wherein said donor host cell is obtained by a method comprising:

combining a prokaryotic cell lacking a DNA sequence encoding said polypeptide of interest with said DNA construct under fusing conditions;

isolating transformed cells;

growing said transformed cells at a non-permissive temperature; and

identifying and isolating transformed cells wherein said DNA construct is integrated into a location on the chromosome other than that in said recipient host cell.

19. A method according to any one of Claims 14-18, wherein said prokaryotic cell is a Bacillus.

20. A method according to Claim 19, wherein said Bacillus is an alkalophilic Bacillus strain or a B. licheniformis strain.

21. A method according to Claim 20, wherein said alkalophilic Bacillus strain is Bacillus novo species PB92 and said B. licheniformis strain is B. licheniformis strain T5.

22. A method according to any one of Claim 14-21, wherein said polypeptide of interest is an enzyme.

23. A method according to Claim 22, wherein said enzyme is a serine protease or an amylase.

24. A method according to Claim 23, wherein said serine protease has at least 70% homology in nucleotide sequence with a proteolytic enzyme encoding gene from Bacillus novo species PB92.

25. A method according to Claim 23 or 24, wherein said serine protease has substantially the following amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-P-A-S-F-S-Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH.

26. A method according to any one of Claims 14-25, wherein said DNA construct is pMAX-4 or pElatB.

27. A method according to any one of Claims 14-26, wherein said DNA sequence is integrated by illegitimate recombination.

28. A method according to any one of Claims 14-26, wherein said DNA sequence is integrated by homologous recombination.

29. A method according to any one of Claims 14-26, wherein said DNA construct further comprises a temperature sensitive origin of replication.

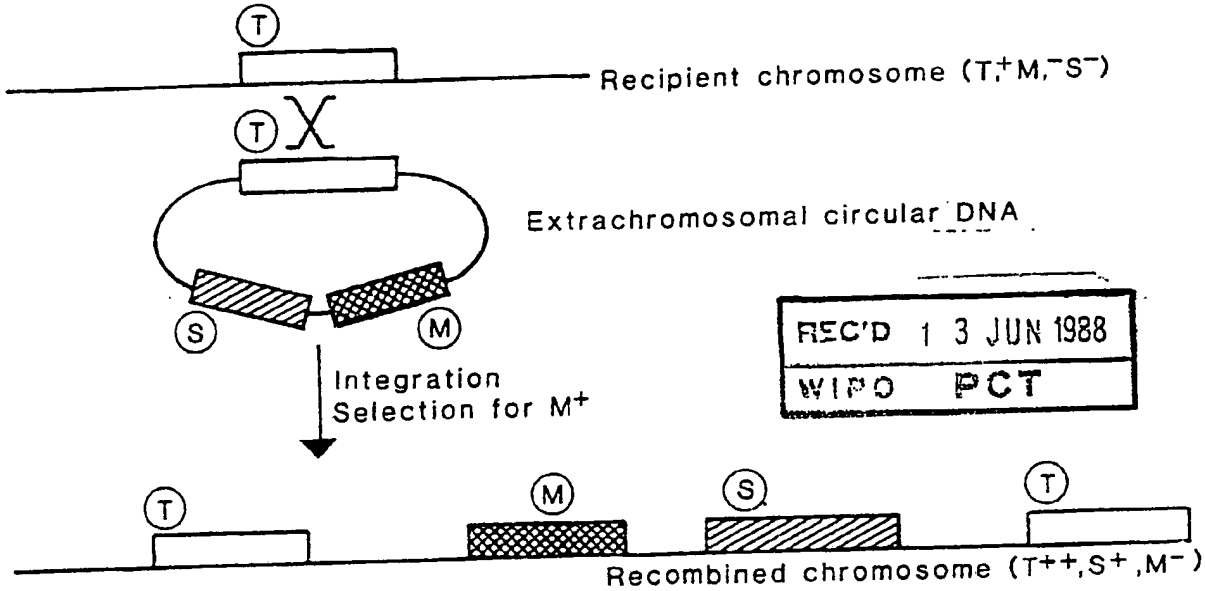
30. A method according to of Claim 29, wherein said

temperature sensitive plasmid is derived from plasmid pE194.

31. Bacillus PBT108, PBT122, or T13F.

5

A. CAMPBELL-TYPE RECOMBINATION



B. DOUBLE RECIPROCAL RECOMBINATION

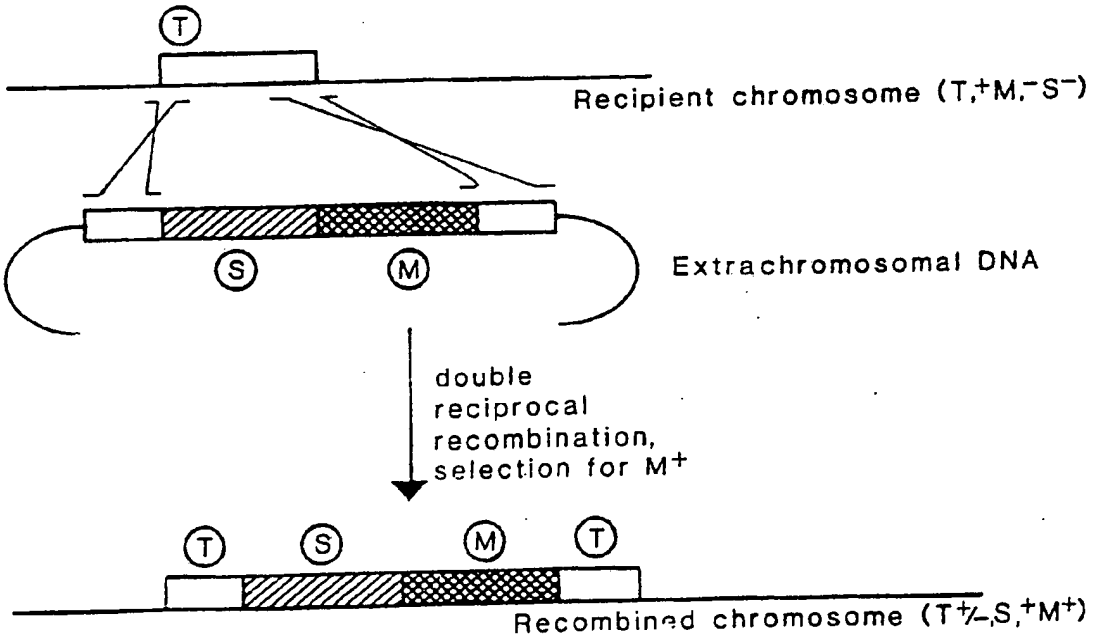
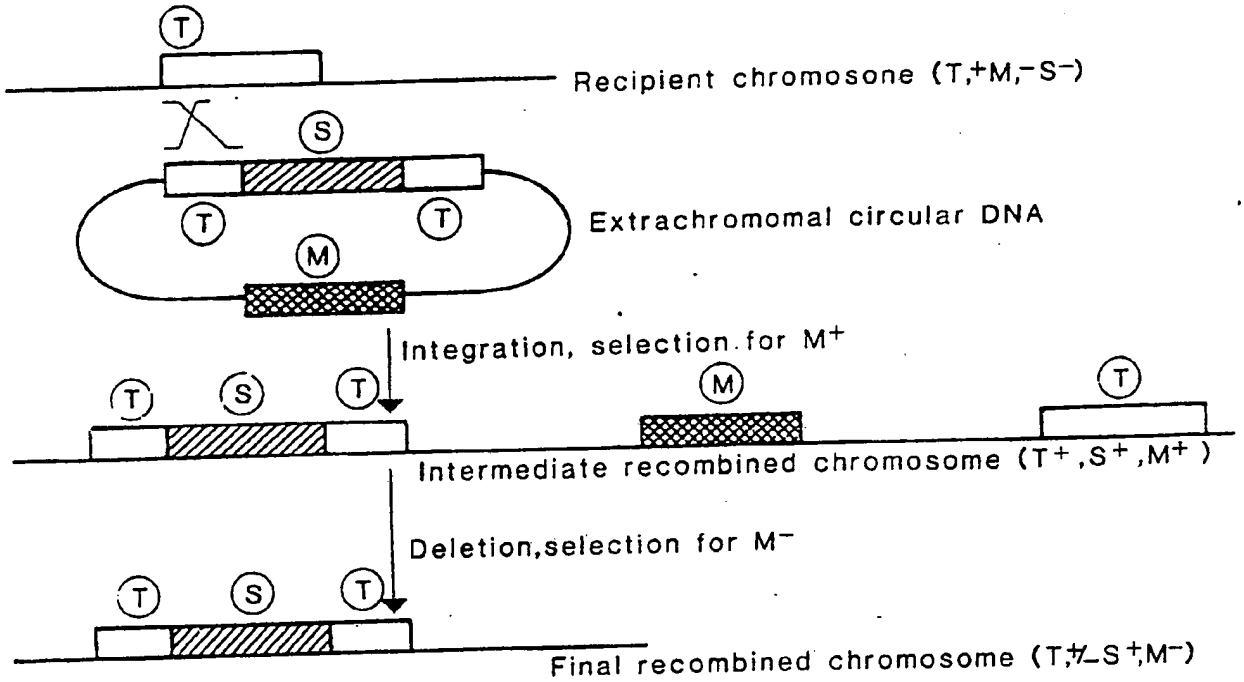


FIGURE 1

C. TWO-STEP INTEGRATION REPLACEMENT MECHANISM



D. INTEGRATION BY ILLEGITIMATE RECOMBINATION

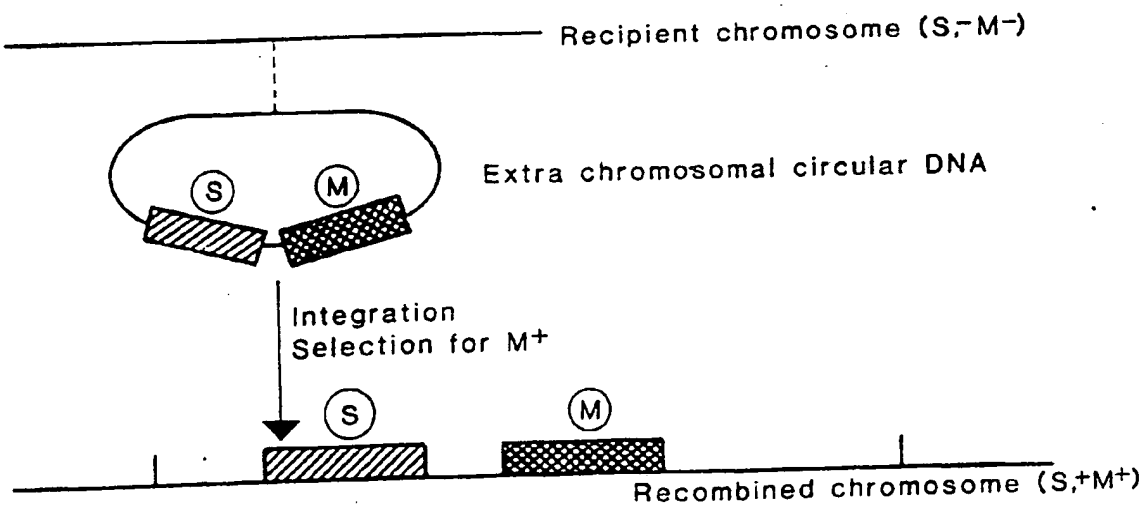


FIGURE 1 (contn'd)

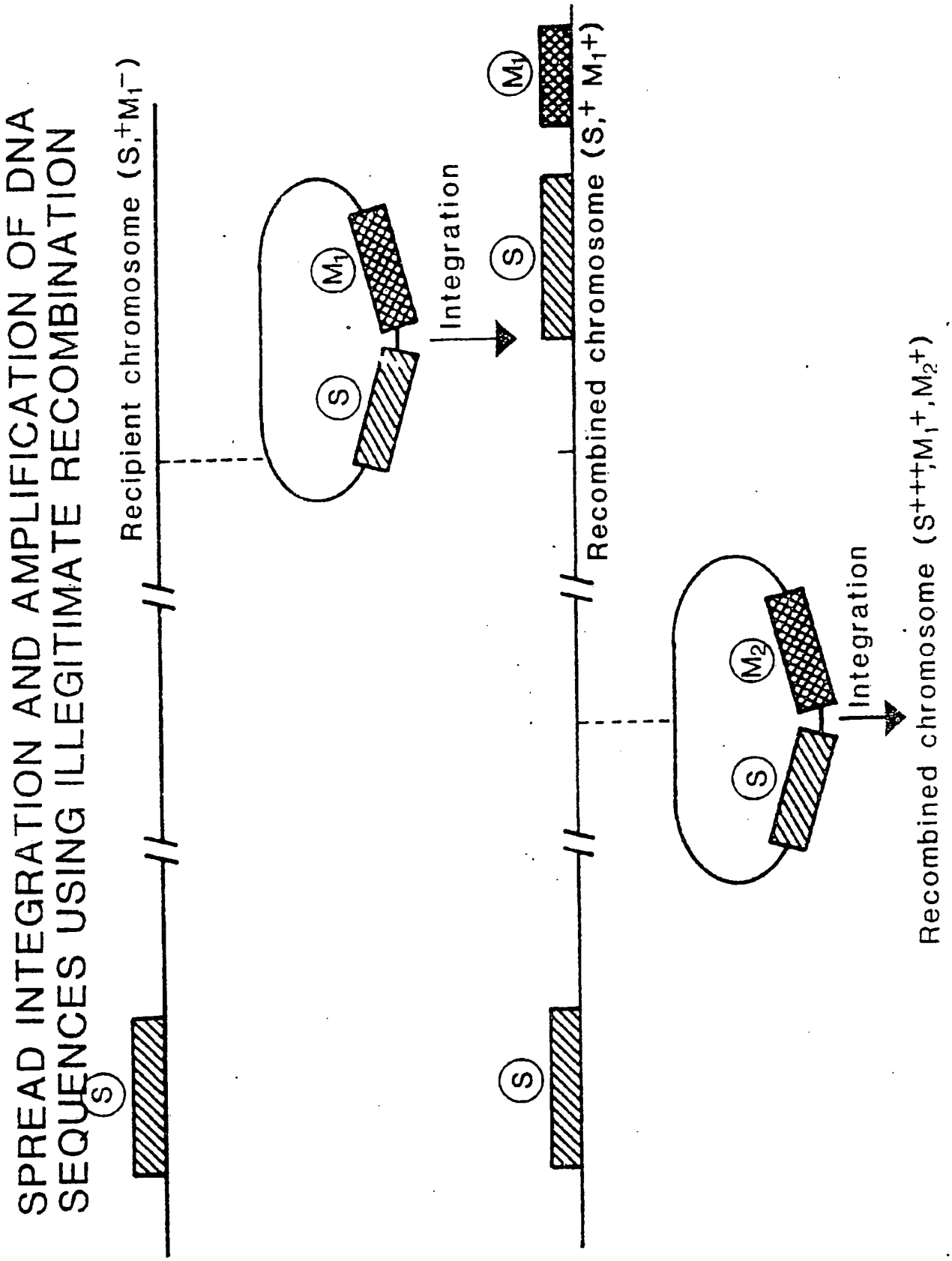

A. SPREAD INTEGRATION AND AMPLIFICATION OF DNA SEQUENCES USING ILLEGITIMATE RECOMBINATION
Recipient chromosome (S,+M1−)
S
M1
S
Integration
M1
S
Recombined chromosome (S,+ M1+)
S
M2
S
Integration
Recombined chromosome (S+++,M1+,M2+)

FIGURE 2

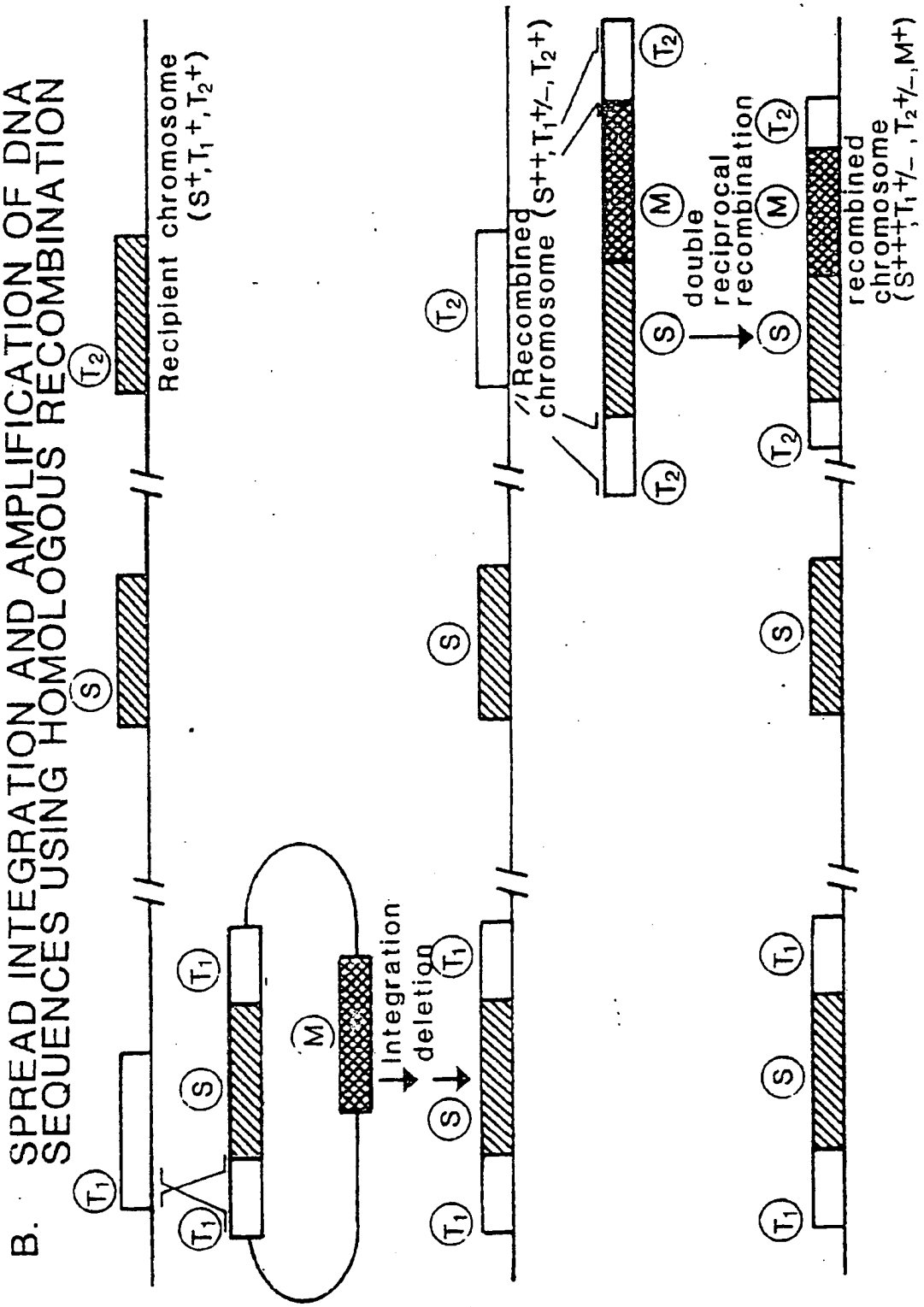


FIGURE 2 (contn'd)

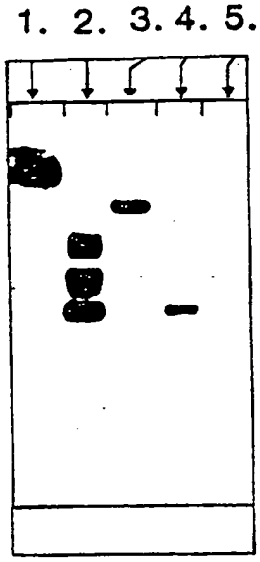


FIGURE 3

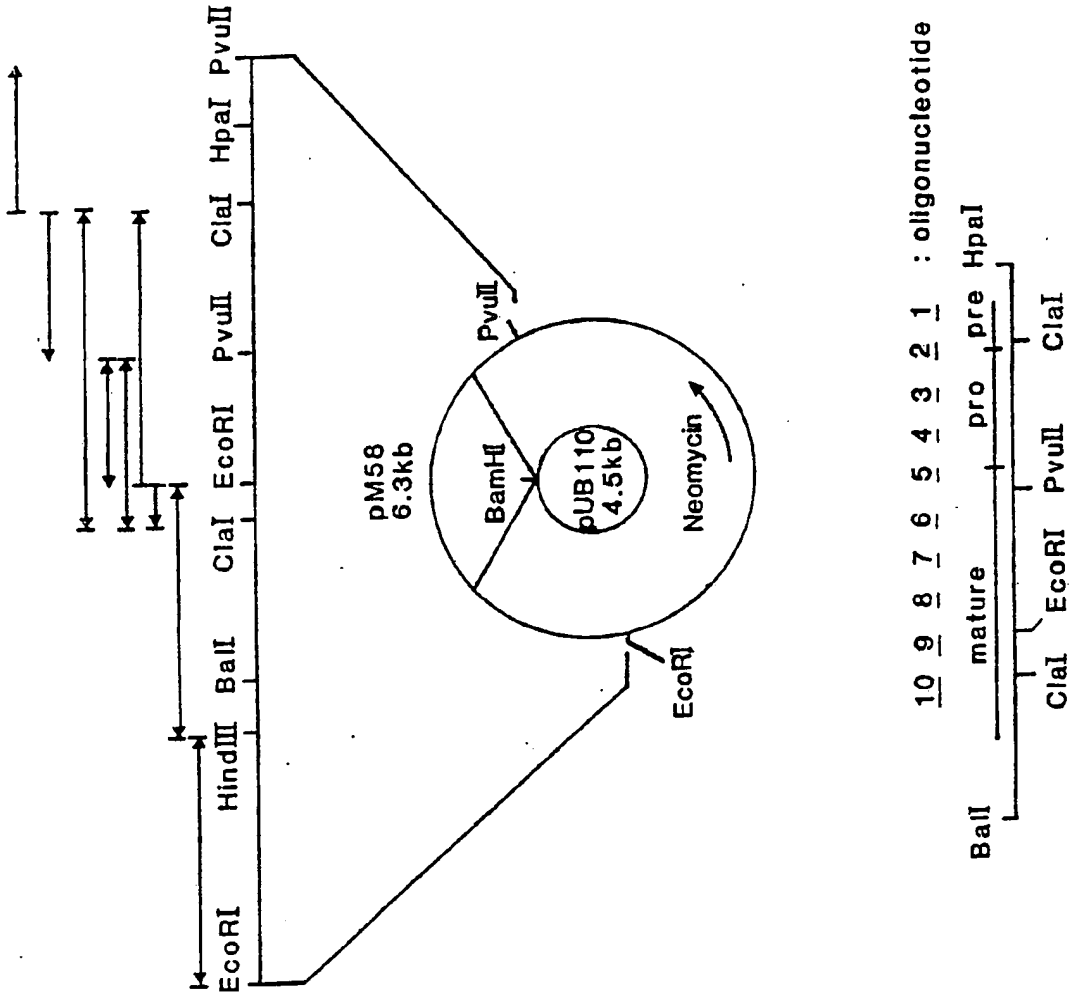

PvuII
HpaI
ClaI
PvuII
EcoRI
ClaI
BalI
HindIII
EcoRI
pM58
6.3kb
PvuII
BamHI
pUB110
4.5kb
Neomycin
EcoRI
10 9 8 7 6 5 4 3 2 1 : oligonucleotide
mature
pro
pre
HpaI
ClaI
EcoRI
PvuII
ClaI
BalI

FIGURE 4

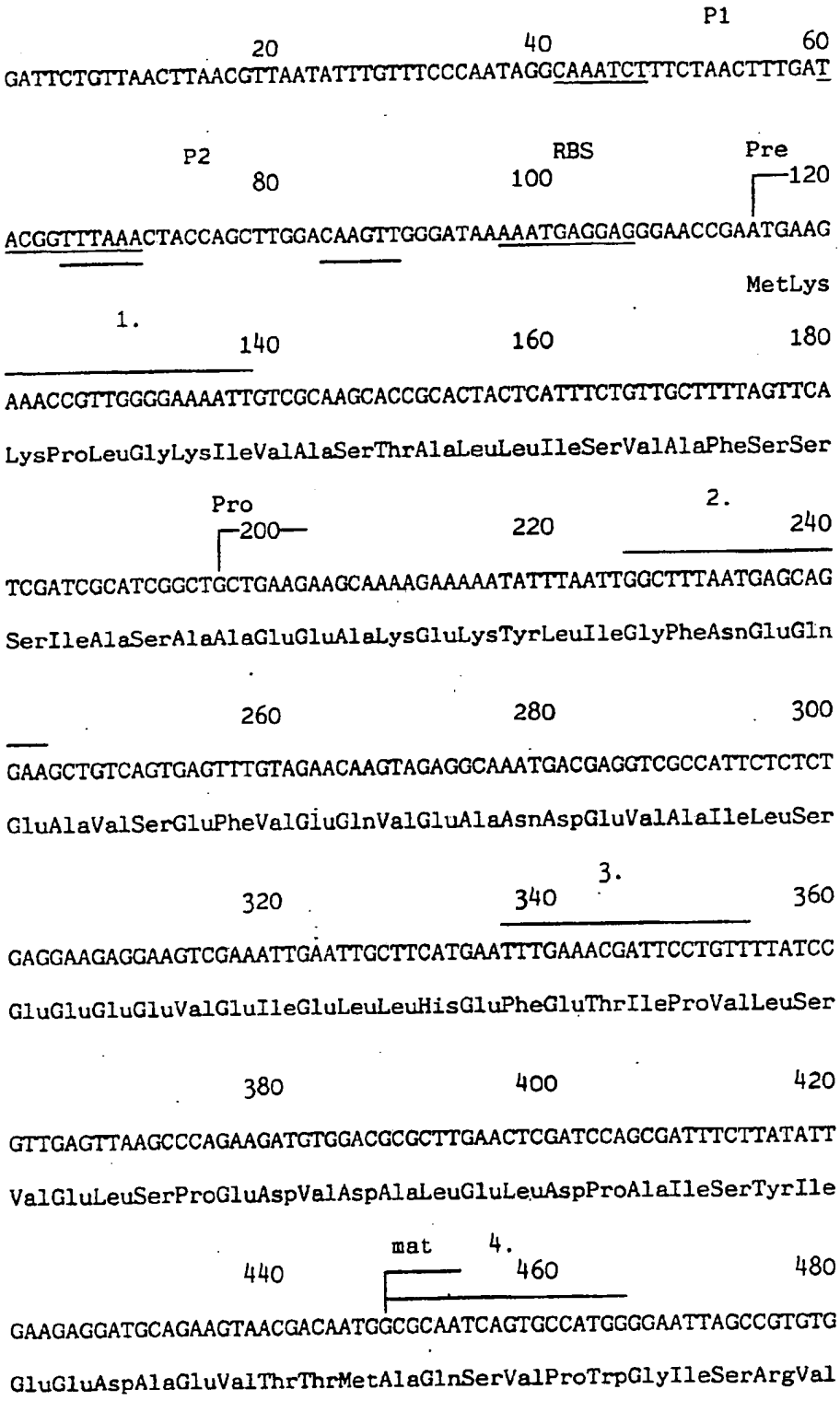

P1
20
40
60
GATTCTGTTAACTTAACGTTAATATTTGTTTCCCAATAGGCAAATCTTTCTAACTTTGAT
P2
RBS
Pre
80
100
120
ACGGTTTAAACTACCAGCTTGGACAAGTTGGGATAAAAATGAGGAGGGAACCGAATGAAG
MetLys
1.
140
160
180
AAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTTAGTTCA
LysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPheSerSer
Pro
2.
200
220
240
TCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAATGAGCAG
SerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsnGluGln
260
280
300
GAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATTCTCTCT
GluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIleLeuSer
3.
320
340
360
GAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTTTTATCC
GluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProValLeuSer
380
400
420
GTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCTTATATT
ValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSerTyrIle
mat
4.
440
460
480
GAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGCCGTGTG
GluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSerArgVal

FIGURE 5

500 520 540

CAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTC

GlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAlaValLeu

5.

560 580 600

GATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCA

AspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPheValPro

620 640 660

GGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCTGGGACGATTGCT

GlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThrIleAla

6.

680 700 720

GCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCACCGAACGCGGAACTATACGCTGTT

AlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProAsnAlaGluLeuTyrAlaVal

740 760 780

AAAGTATTAGGGGCGAGCGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTGGAATGG

LysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeuGluTrp

7.

800 820 840

GCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCC

AlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerProSerAla

860 880 900

ACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCGGCATCT

ThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAlaAlaSer

8.

920 940 960

GGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTC

GlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMetAlaVal

FIGURE 5 (contn'd)

980 1000 1020

GGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGAC

GlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGlyLeuAsp

9.

1040 1060 1080

ATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCCAGCTTA

IleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAlaSerLeu

10.

1100 1120 1140

AACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAACAAAAG

AsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLysGlnLys

1160 1180 1200

AACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACGAGCTTG

AsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThrSerLeu

1220 1240 1260

GGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGCTAATCA

GlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

1280 1300 1320

ATAAAAACGCTGTGCTTAAAGGGCACAGCGTTTTTTTGTGTATGAATCGAAAAAGAGAAC

terminator

FIGURE 5 (contn'd)

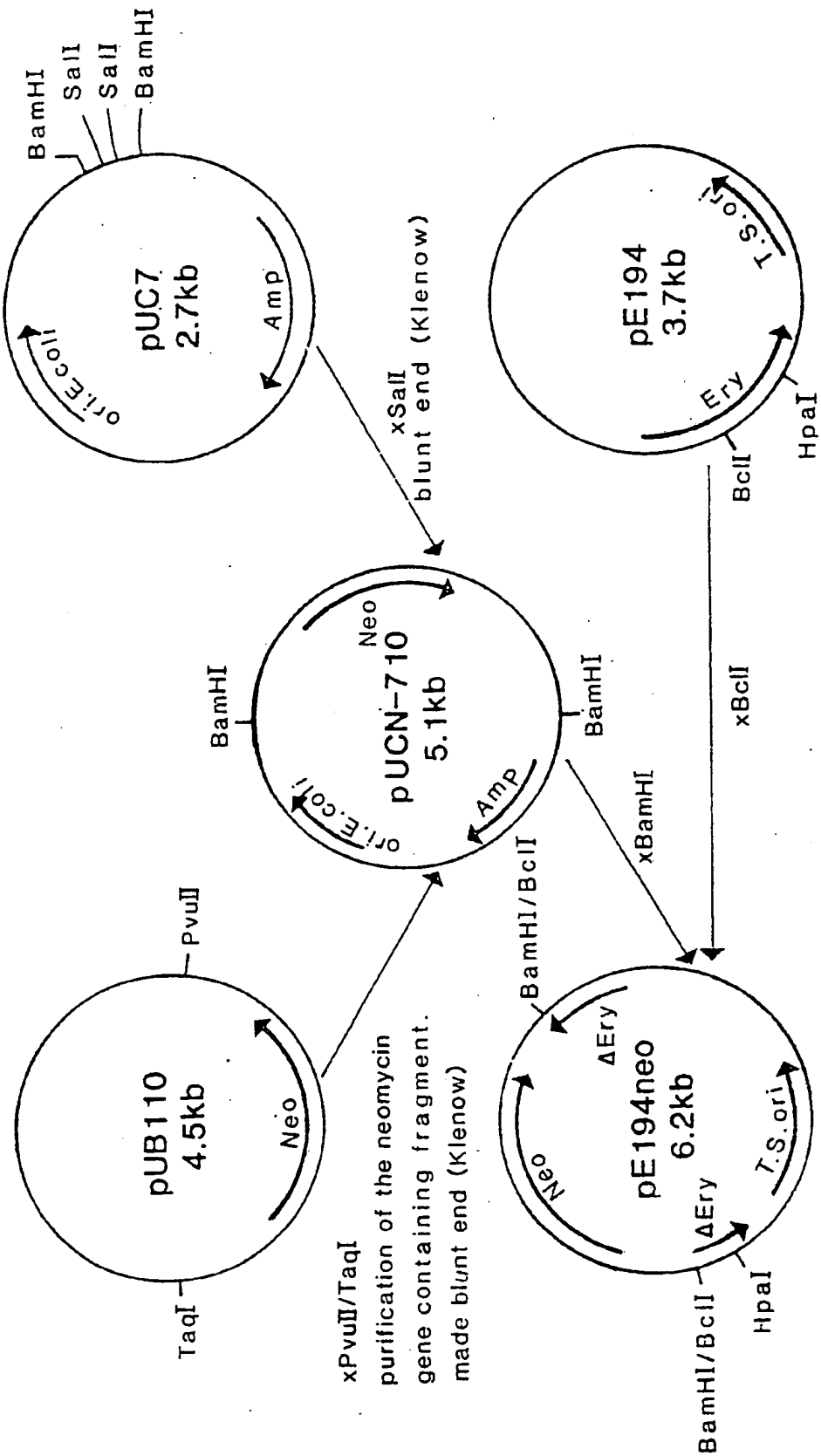

BamHI
SalI
SalI
BamHI
pUC7
2.7kb
Amp
ori.E.coli
xSalI
blunt end (Klenow)
pE194
3.7kb
Ery
T.S.ori
BclI
HpaI
xBclI
Neo
pUCN-710
5.1kb
Amp
ori.E.coli
BamHI
BamHI
xBamHI
PvuII
pUB110
4.5kb
Neo
TaqI
xPvuII/TaqI
purification of the neomycin
gene containing fragment.
made blunt end (Klenow)
BamHI/BclI
ΔEry
pE194neo
6.2kb
Neo
ΔEry
T.S.ori
BamHI/BclI
HpaI

FIGURE 6A

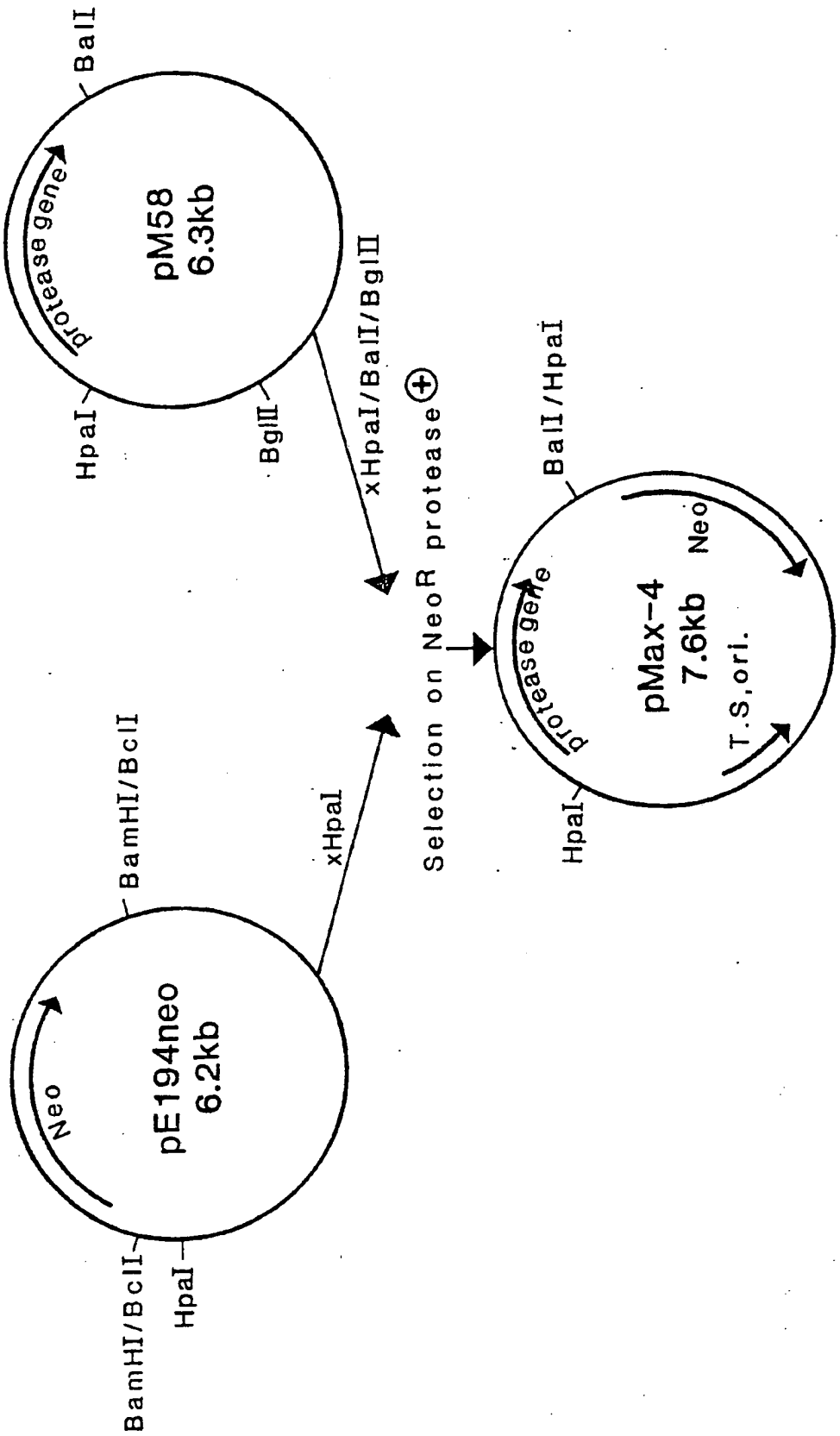
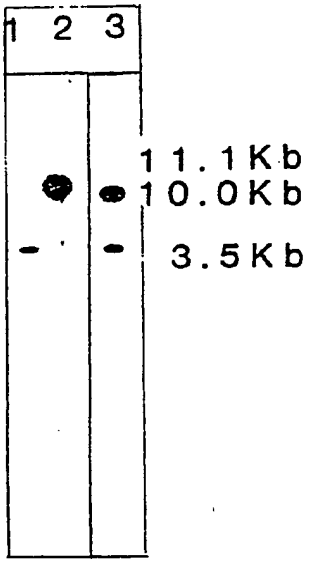

BalI
pM58
6.3kb
protease gene
HpaI
BglII
xHpaI/BalI/BglII
Selection on NeoR protease
BalI/HpaI
pMax-4
7.6kb
protease gene
Neo
T.S.ori.
HpaI
BamHI/BclI
pE194neo
6.2kb
Neo
BamHI/BclI
HpaI
xHpaI

FIGURE 6B



Lane 1: PB92

Lane 2: PBT 109

Lane 3: PBT 108

FIGURE 7A

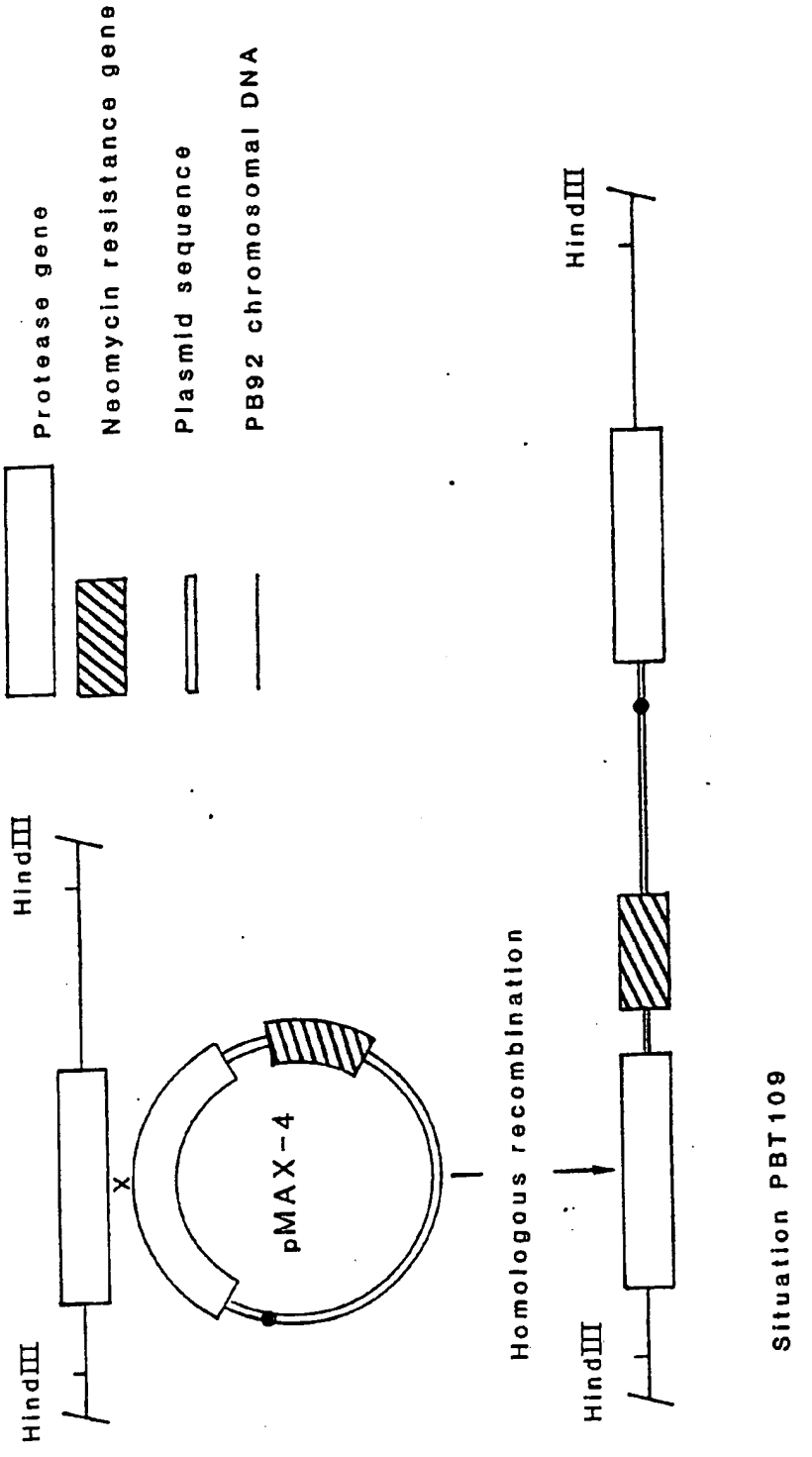

Protease gene
Neomycin resistance gene
Plasmid sequence
PB92 chromosomal DNA
HindIII
HindIII
pMAX-4
Homologous recombination
HindIII
HindIII
Situation PBT109

FIGURE 7B

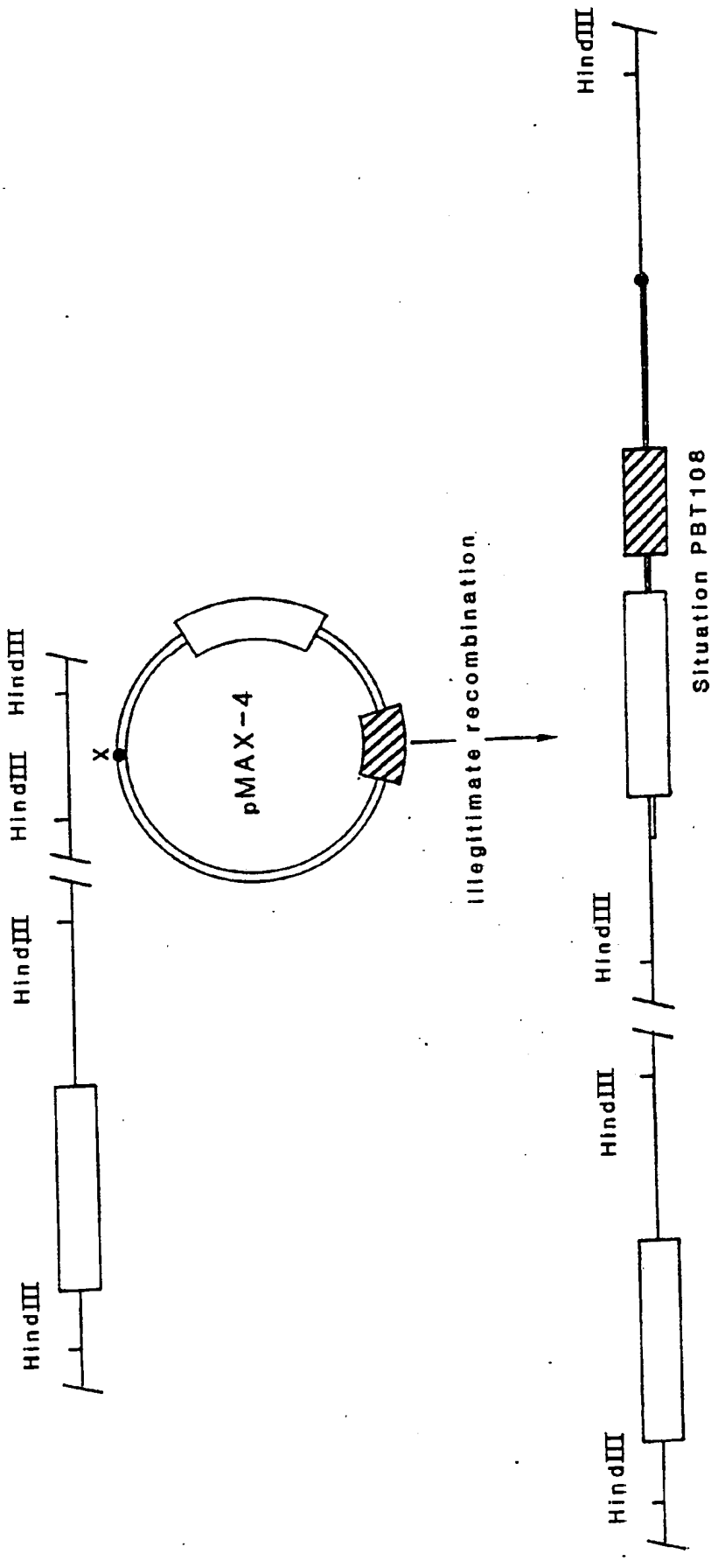

HindIII
HindIII
HindIII
X
pMAX-4
HindIII
HindIII
Illegitimate recombination
HindIII
HindIII
HindIII
HindIII
Situation PBT108

FIGURE 7C

CONSTRUCTION OF INTEGRATION VECTOR pELATB

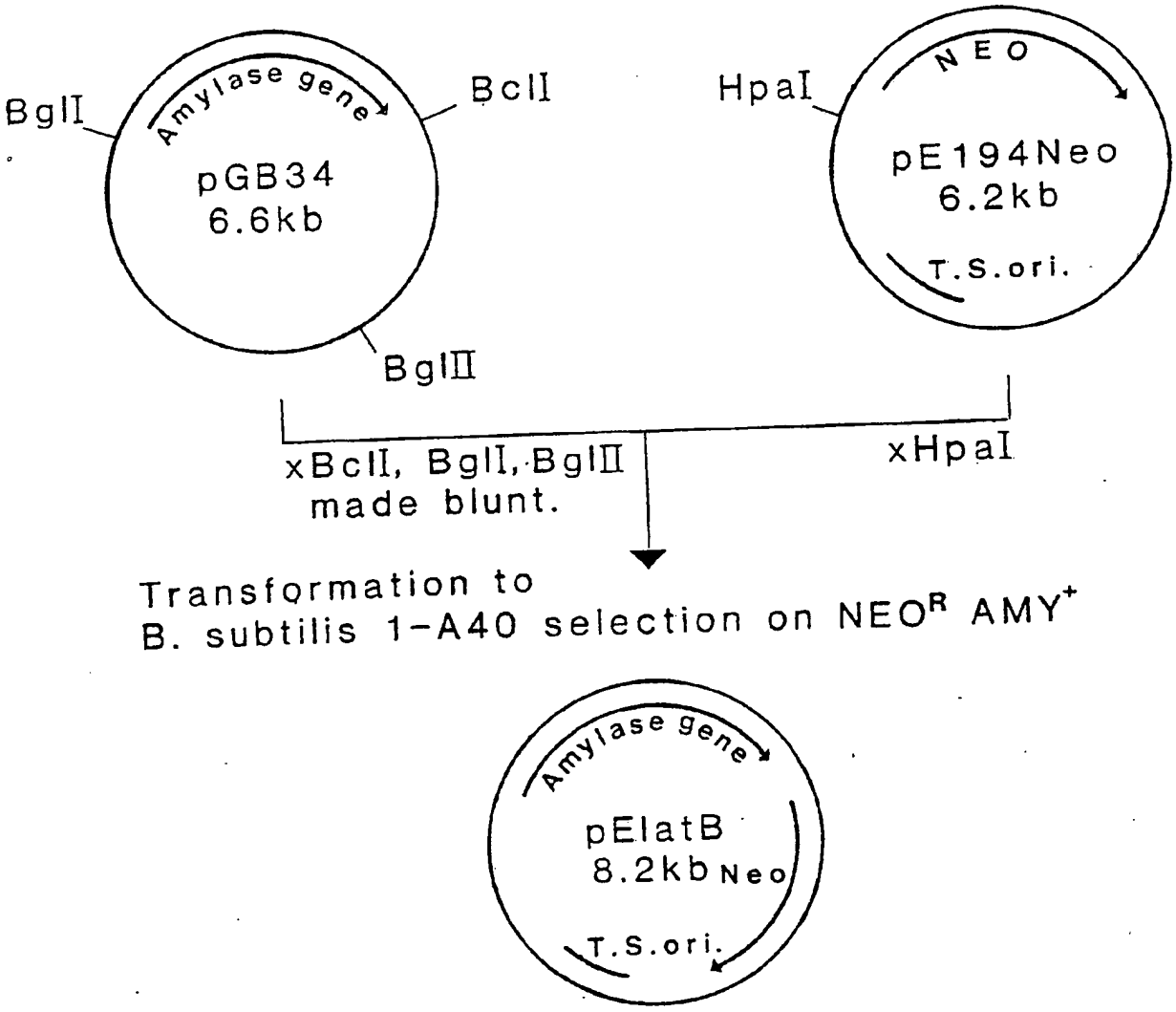


FIGURE 8

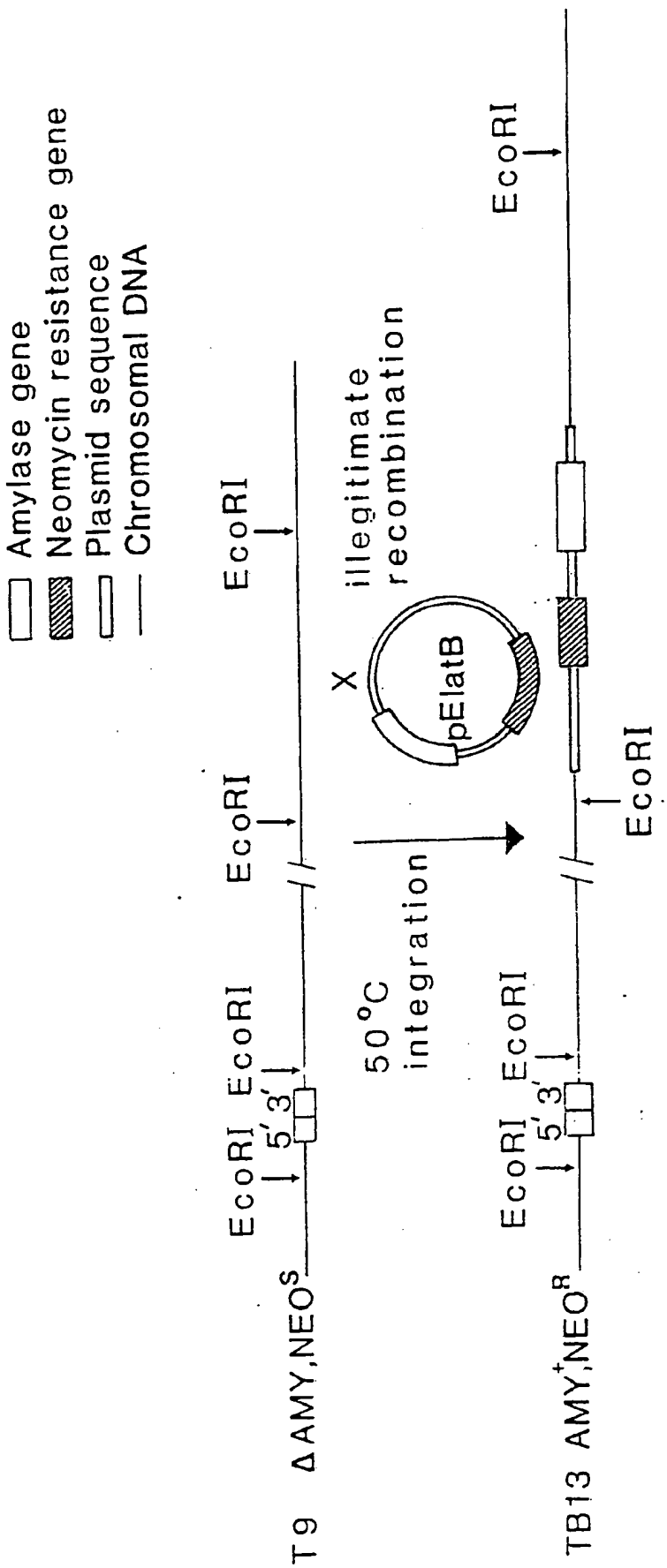

Amylase gene
Neomycin resistance gene
Plasmid sequence
Chromosomal DNA
EcoRI
EcoRI
illegitimate recombination
X
pElatB
EcoRI
EcoRI
50 °C integration
EcoRI EcoRI
5' 3'
T9 ΔAMY,NEO^S
EcoRI EcoRI
5' 3'
TB13 AMY^+,NEO^R

FIGURE 9A

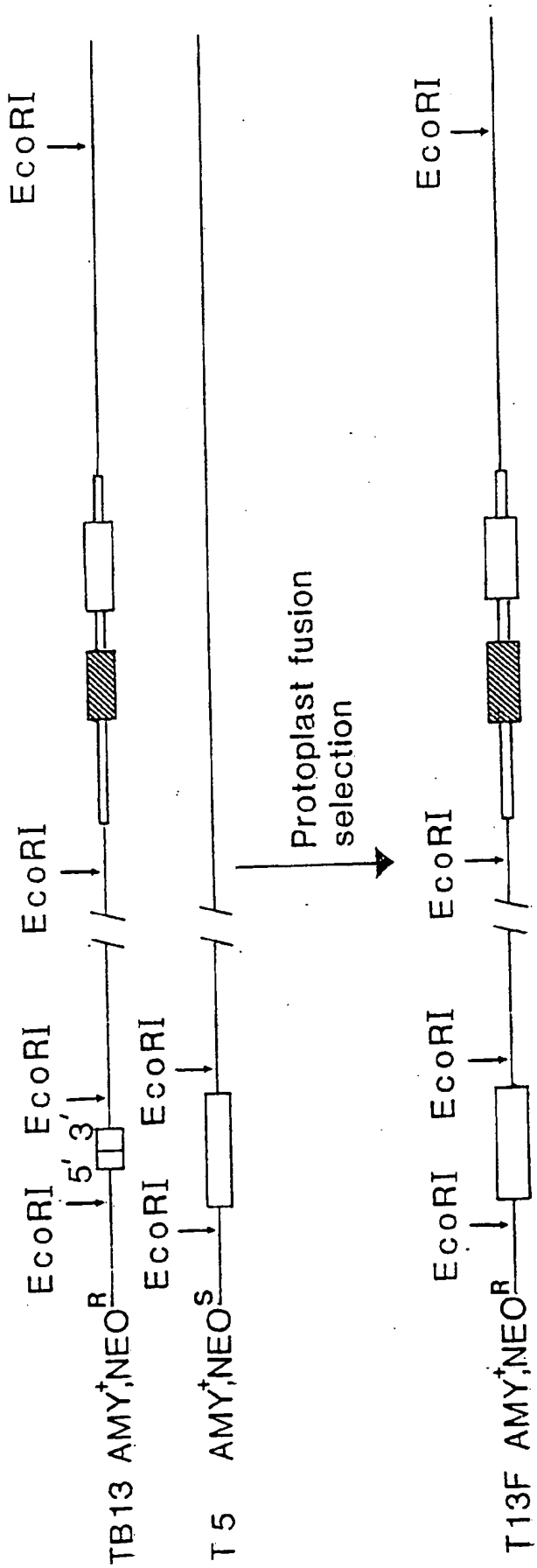

EcoRI
EcoRI
EcoRI
5'
3'
EcoRI
EcoRI
EcoRI
TB13 AMY+,NEOR
T5 AMY+,NEOS
Protoplast fusion
selection
EcoRI
EcoRI
EcoRI
EcoRI
T13F AMY+,NEOR

FIGURE 9B

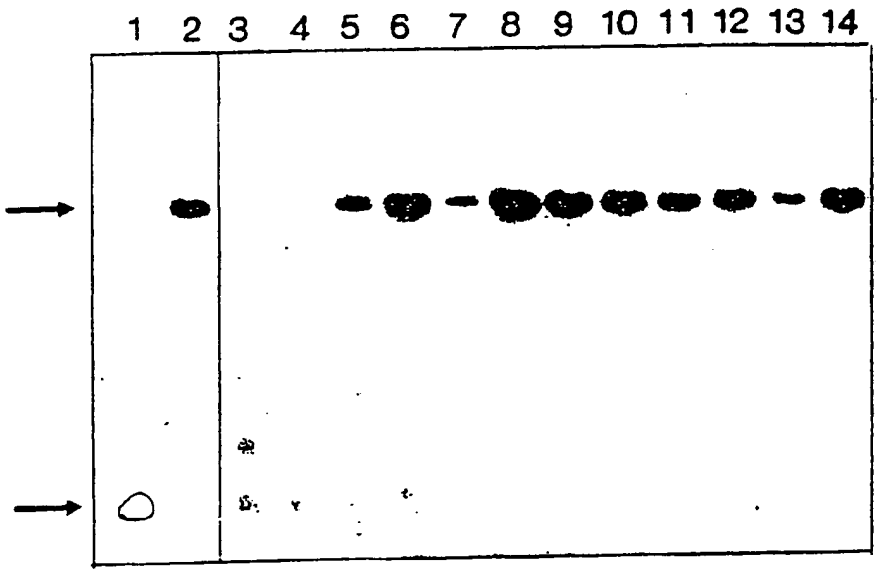


FIGURE 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 88/00006

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 1/20// C 12 N 9/54

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0124374 (GENEX) 7 November 1984, see claims 1-4	1,2,3
Y	see claims 10,11; page 8, lines 10-25	4,6-9,11,13
X	EP, A, 0205371 (CNRS) 17 December 1986, see column 9, line 8; page 8, table, results QB118::(pCG1)	1-3,7,8,11
X	Chemical Abstracts, vol. 98, 1983, (Columbus, Ohio, US), J. Hofemeister et al.: "Integration of plasmid pE194 at multiple sites on the Bacillus subtilis chromosome", see page 88, abstract no. 155686a, MGG, Mol. Gen. Genet. 1983, 189(1), 58-68	1
A		14-16
X	EP, A, 0127328 (GENEX) 5 December 1984, see page 6, line 29 - page 7, line 7; page 7, lines 23-29; page 19, lines 6-11; page 19, line 34 - page 20, line 19 ./.	1-3,7,8,11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th June 1988	01 AUG 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	--	14-24,27-30
X	EP, A, 0074553 (UNIVERSITY OF ROCHESTER) 23 March 1983, see page 16, line 34 - page 18, line 4 --	1-3,7,8,11
Y	EP, A, 0134048 (GIST BROCADES) 13 March 1985, see the whole document --	4,6-9,11, 13,14-24, 27-30
X	Chemical Abstracts, vol. 108, 1988, (Columbus, ohio, US), P. Kallio et al.: "Enhancement of alpha-amylase production by integrating and amplifying the alpha-amylase gene of Bacillus amyloliquefaciens in the genome of Bacillus subtilis", see abstract no. 88925n, Appl. Microbiol. Biotechnol. 1987, 27(1), 64-71 --	1,2,3,7,8, 11
A	WO, A, 86/01825 (GENEX) 27 March 1986, see page 10, line 15 - page 13, line 27; figure 2; page 16, lines 1-20 --	14-19,22,23
A	FR, A, 2292042 (GIST BROCADES) 18 June 1976 --	10,24,25
A	EP, A, 0130756 (GENENTECH) 9 January 1985, see page 4, lines 28-35; page 6, lines 21-25; page 10, lines 26-35; page 13, lines 1-8; pages 24-29; figures 1,7 --	7,8,9,10, 22,23,24, 25
A	Biological Abstracts, vol. 82, 1986, Biosciences Information Service (Philadelphia, US), J.H. Yu et al.: "Physiological properties and transformation of alkaline-tolerant bacteria", abstract no. 86427, J. Appl. Microbiol. bioeng 14(3): 239-244, 1986 --	4,20
A	EP, A, 0032238 (UNIVERSITY OF ROCHESTER) 22 July 1981, see page 8, lines 1-13 --	
A	Gene, vol. 24, 28 September 1983, Elsevier Science Publishers (Amsterdam, NL), J.G.K. Williams et al.: "Stable integration of foreign DNA into the chromosome of the cyanobacterium Synechococcus R2", pages 37-51, see abstract; page 49, last paragraph --	
E	WO, A, 87/04461 (AMGEN) 30 July 1987, see page 30, lines 3-6 ------	1-3,7,8,9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 8800006 SA 21025

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 25/07/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0124374	07-11-84	JP-A- 59205996	21-11-84
		US-A- 4617266	14-10-86
EP-A- 0205371	17-12-86	FR-A- 2582316	28-11-86
EP-A- 0127328	05-12-84	JP-A- 59205983	21-11-84
EP-A- 0074553	23-03-83	JP-A- 58067186	21-04-83
EP-A- 0134048	13-03-85	WO-A- 8500382	31-01-85
		AU-A- 3151084	07-02-85
		AU-B- 570709	24-03-88
WO-A- 8601825	27-03-86	EP-A- 0195078	24-09-86
		JP-T- 62500215	29-01-87
FR-A- 2292042	18-06-76	NL-A- 7513433	21-05-76
		LU-A- 73826	11-06-76
		BE-A- 835693	18-05-76
		DE-A,B,C 2551742	26-05-76
		US-A- 4002572	11-01-77
		GB-A- 1519148	26-07-78
		JP-A- 51082783	20-07-76
		JP-A- 51125407	01-11-76
		JP-A- 55148086	18-11-80
		US-E- 30602	05-05-81
		CH-A- 626916	15-12-81
		SE-A- 8104191	06-07-81
		SE-A- 7512938	19-05-76
		SE-B- 435396	24-09-84
		SE-B- 442749	27-01-86
EP-A- 0130756	09-01-85	AU-A- 2956884	03-01-85
		JP-A- 60070075	20-04-85
		EP-A- 0246678	25-11-87
		EP-A- 0247647	02-12-87
EP-A- 0032238	22-07-81	JP-A- 56113293	07-09-81
		JP-A- 62163694	20-07-87

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 8800006 SA 21025

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 25/07/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0032238		None	
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